

PLATELET ADHESION IN ATHEROGENESIS

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1. Abstract

Platelets are known to play a key role in acute coronary events, such as myocardial infarction, associated with advanced atherosclerosis, but a number of papers have recently been published suggesting that platelet adhesion may initiate atherosclerotic lesion formation. The proposed research aimed to investigate this by seeing whether platelet adhesion occurred, directly or via leukocytes, to the endothelium in diabetic conditions. Diabetic-like conditions were chosen because an accelerated rate of lesion formation occurs. This was achieved by incubating endothelial cells with high concentrations of glucose and various types of AGEs.

Bovine serum albumin of different purity, a peptide of albumin, and haemoglobin were glycated with D-glucose. Samples were taken at 2 weeks intervals to allow for determining the extent of glycation and allowing for investigation into whether the extent of glycation affected platelet adhesion. It also made possible analysis of extent of glycation, to see whether these have a relation to endothelial dysfunction (including cell proliferation, cell adhesion molecule expression, and ROS and cytokine production). There are discrepancies in the finding of studies looking into the effect of AGEs on the endothelium and there are no reported studies looking at cytokine production. As such, it was hoped that this research would allow for a greater understanding of the processes involved and whether endothelial dysfunction could account for accelerated lesion formation associated with diabetes.

As a whole, the research intended to explore the hypothesis that platelet adhesion to the endothelium is required for foam cell formation and their development into atherosclerotic lesions. It aimed to see whether hyperglycaemia and the presence of high levels of AGEs induces endothelial dysfunction and therefore increases monocyte adhesion by the presence of activated platelets. In doing so, a greater understanding of the processes involved in accelerated lesion formation would allow for targeted research to allow for treatments to reduce foam cell deposition. This could be the use of a cocktail of anti-platelet drugs or ones that reduce endothelial dysfunction. This would reduce the manifestation of cardiovascular disease and therefore improve diabetic patient life as well as reduce the cost of treatment for the NHS, and therefore for the tax payer.

2. Introduction

Atherosclerosis is prevalent in the western world and accounts for high levels of both mortality and morbidity. Although a lot is known about the processes involved in this vascular disease, there are still aspects that are not clearly defined and areas where scientific opinions differ.

2.1. Atherosclerosis

Atherosclerosis is a form of macrovascular disease that is characterized by arterial fatty deposits that cause reduced elasticity of the blood vessel. The word was first coined by the German Pathologist, Felix Marchand, in 1904 from the Latin for gruel hardening, although Joseph Lobstein has previously used the term arteriosclerosis for describing the general loss of elasticity of blood vessels back in 1833 and Albrecht von Haller used the word atheroma in 1755 to describe the accumulation of cells in the blood vessel (Gotto, 2005, Tedgui and Mallat, 2006). Atherosclerosis had, however, been described before this. For instance, Leonardo Da Vinci detailed the thickening of arteries with age in the 1400s (Ose, 2008). Since the 1800s, there has been a wealth of research that has characterised the development and causes of this disease.

In the 1890s, Karl von Rokitansky and Rudolf Virchow set out their opposing theories on atherosclerosis. Rokitansky believed that lesions developed from deposition of small blood clots within the intima. This led to infiltration of fibroblasts and deposition of lipids. Virchow, on the other hand, believed that lipid deposition, intimal injury and inflammation were key (Mayerl et al., 2006). Virchow's observations have been added to and form the basis of our understanding of this disease.

Atherosclerosis is divided into a number of stages; these are determined by the stability of the plaque and by the state of the endothelium (see Table 1). The initial phase of atherosclerotic plaque formation, fatty deposits, occurs from early childhood (Jarvisalo et al., 2001, Napoli et al., 1997) which concurs with Da Vinci that atherosclerosis is part of the general aging process. These fatty deposits start as fatty streaks. Fatty streaks develop when monocytes engulf oxidised low-density lipids (ox-LDL) and migrate through the endothelium and into the intima of the blood vessel, becoming lipid-rich macrophages or foam cells. The macrophages are unable to process these lipids and therefore they are eventually dispersed resulting in the accumulation of more monocytes.

Intermediate lesions occur from the accumulation of foam cells to form a layer on the artery wall. This results in proliferation and migration of smooth muscle cells. The lesion can eventually develop to form fibrous plaque that causes narrowing of the blood vessel and associated ischaemia. This is a complex lesion that consists of a cap and core. The cap is formed from muscle and endothelial cells and results in hardening of the vessel. The core is formed from foam cells, muscle cells, and general cellular debris – giving the

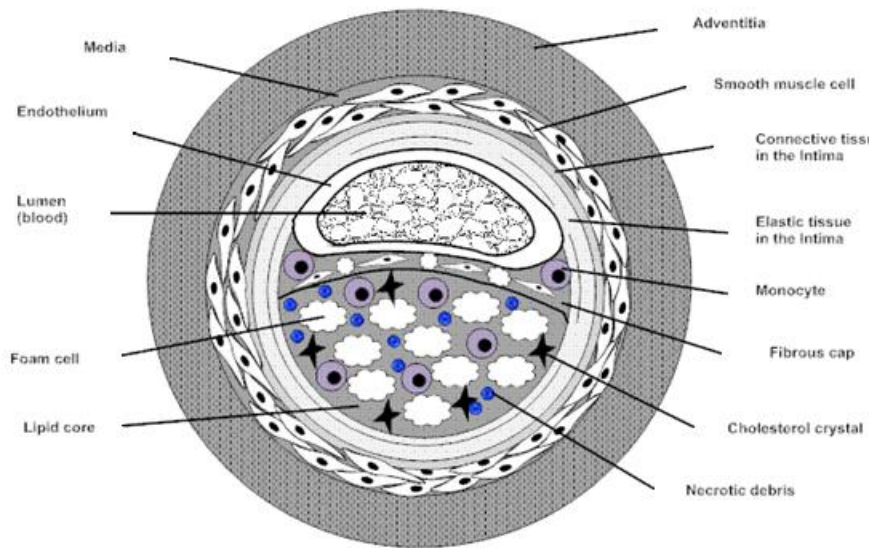
lesion gruel like appearance. These complex lesions are prone to become unstable and rupture, resulting in thrombus formation and the associated pathologies of stroke, gangrene, and myocardial infarcts (Ross, 1999a, Stary et al., 1995, Stary et al., 1994).

Table 1: The Stages of Atherosclerosis (From the Findings of Stary et al. 1994 & 5)

Type	Description	Details
I	Initial stage of atherosclerosis	Lipid deposition in the intima Invisible to the naked eye Isolated FCs
II	Fatty streak (initial)	Accumulation of FCs Visible as yellowy streaks / areas Stains red with Sudan (III/IV) dye SMCs contain lipid droplets T lymphocytes and mast cells present Increased number of macrophages Lipid visible in the extracellular space
III	Intermediate lesion	Lipid pools Lipids present in extracellular space and SMCs Increased levels of cholesterol, fatty acids, sphingomyelin, lysolecitin, and triglycerides
IV	Atheroma (advanced)	Lipid core Fibrous cap (macrophages, FCs, SMCs) Endothelium still intact No narrowing of the lumen Lipid accumulation in the extracellular space Increased lymphocytes and mast cells
V	Fibroatheroma (advanced)	Narrowing of the lumen Fibrous connective tissue formation Lipid core calcification Vascularization Fissures possibly present – thrombus formation
VI	Complicated lesion (advanced)	Significant narrowing of the lumen Disruption to the lesion surface Thrombotic deposits (haematoma / haemorrhage)

(Key: FCs - foam cells; SMCs - smooth muscle cells)

Figure 1: Atherosclerosis



At present, the processes involved in initiating atherosclerosis are not fully understood. In 1973, Ross and Glomset suggested that lipid deposition occurred as a result of injury to endothelial cells, with endothelial denudation occurring prior to atheroma formation (Ross and Glomset, 1973). This was refined by Williams and Tabas (Williams and Tabas, 1998). They stated that there was no clinical evidence to suggest that endothelial denudation took place until latter stages of atherosclerosis. In vivo evidence suggested that an intact endothelium was necessary for the initial development of lesions. Their 'response-to-retention' theory suggested that endothelial functional changes took place, but these were not sufficient for denudation. These functional changes were thought to be due to a number of possible factors including decreased production of the vasodilators nitric oxide (NO) and prostacyclin (PGI₂), inactivation of NO and/or increased production of endothelium-derived contracting factors, cytokine production, cell adhesion molecule activation, and reactive oxygen species (ROS) formation. The results of these changes are that there is increased lipoprotein retention in endothelial cells leading to plaque formation.

More recently, research has centred on the role of progenitor cells in atherogenesis (Daub et al., 2006, Daub et al., 2007, Xu et al., 2008). It has been postulated that because atherosclerosis increases with age it may result from the reduced capacity for, or dysfunction in, cell regeneration. Research has highlighted some evidence for linking endothelial cell repair mechanisms with lesion formation. Indeed, Xu and colleagues (2008) reported that endothelial apoptosis occurred in the lead up to lesion formation in a mouse model of atherosclerosis. They also showed CD31- positive cells a week after injury inducement by cuff placement, prior to visible lesions at 2 weeks, suggesting that

progenitor cells were involved. It is clear, therefore that changes to the endothelium are involved in atherogenesis.

2.2. Endothelium

The endothelium acts as a semi-permeable barrier between the blood and the surrounding tissue. It is actively involved in regulating the vascular tone and hence the supply of blood and nutrients to the surrounding tissue. Under normal physiological conditions the endothelium maintains vasomotor control and an antithrombotic state.

Blood vessels comprise a number of layers or tunics: the adventitia, media, and intima, with the lumen making up the channel for blood transport. The adventitia is formed from dense connective tissue, which consists of elastin and collagen fibres. Nerves, small diameter vessels (vasa vasorum) and lymphatic vessels impregnate this layer. This forms the backbone of the blood vessels, allowing for rigidity, support, contraction, and for communication (Laflamme et al., 2006). The tunica media is formed from smooth muscle cells and elastin. These allow for maintenance of the vasomotor control and its thickness is dependent on the function of the vessel. For instance, muscular arteries have a thick media tunica to allow for diameter changes in response to the blood requirements of the target tissue (Avolio et al., 1998). The intima consists of a monolayer of endothelial cells, held extremely closely together with tight junctions, and another layer, called the basement layer, which is formed from elastin and collagen, as well as other cells, including fibroblasts, macrophages and smooth muscle cells (Millonig et al., 2001). The endothelium is primarily involved in maintaining a non-thrombotic surface and providing a semi-permeable barrier between the blood vessels and the blood. The endothelium therefore has a major role in blood vessel function.

The endothelial cell monolayer is approximately 0.5 - 1.2 μm thick (Hoak et al., 1981) and is held together so tightly that under the microscope the cells look as if they overlap. The individual cells elongate in the direction of the blood flow and therefore provide a very large surface area to volume ratio. This allows for maximal diffusion of small macromolecules, leukocytes and fluid, which is essential for the health and wellbeing of the underlying cells.

The endothelium is negatively charged, as are platelets (Sawyer and Srinivasan, 1972). Therefore repulsion of platelets is achieved in normal physiological conditions. This forms the basis of the vessel wall non-thrombogenic surface. The endothelium, however, also has the ability to prevent platelet adhesion and coagulation in other ways, such as the production of heparin-like molecules, ecto-ADPase, nitric oxide and prostacyclin (PGI_2), and the binding and inactivation of circulating thrombin (Hoak et al., 1981, Sneddon and Vane, 1988, Venturini et al., 1989).

Heparin-like molecules bind circulating antithrombin. Circulating thrombin is anchored to form thrombin-antithrombin complexes. The anchoring of thrombin results in this

compound not being available for fibrinogen cleavage. This process results in inactivation of a number of coagulation factors, including Xa and IXa, therefore the clotting cascade and clot formation is inhibited (Frebelius et al., 1996, Marcum and Rosenberg, 1984).

The production of ecto-ADPase (CD39) by the endothelium has a major role in haemostasis. The conversion of ADP to AMP is catalysed by this enzyme. ADP is a platelet agonist and, as previously stated, is involved in platelet aggregation and thrombus formation (Gayle et al., 1998, Marcus et al., 1997).

The production of vasodilators such as PGI₂ and NO reduces platelet activation by increasing the concentrations of cAMP and cGMP and therefore the chances of circulating platelet interaction with the vessel wall. These cyclic nucleotides activate protein kinases, cyclic GMP-dependent protein kinase G (PKG) and cyclic AMP-dependent protein kinase A (PKA). PKG is known to inhibit platelet adhesion, partly via decreased production of type 3 phosphodiesterase (Wang et al., 1998) and it is also involved in inactivation of a number of pathways associated with platelet activation (den Dekker et al., 2002).

It is clear that endothelial cells play a role in the maintenance of a 'normal' physiological state, but the endothelium can also be in a state of dysfunction. Cell permeability can be altered and the anti-thrombotic surface can become pro-adhesive to circulating platelets. Endothelial cells have the ability to synthesise and express the vasoconstrictor endothelin-1, a number of cell adhesion molecules, cytokines and chemokines that can be involved in platelet and leukocyte adhesion as well as an immune response, endothelial and smooth muscle cell chemotaxis and proliferation, and cell migration.

2.3. Platelets

Platelets are small (approximately 2 µm in diameter) anuclear blood cells. They are colourless, with a regular discoid shape and are present in the plasma of humans at a concentration of 150 – 400 x 10⁹ per litre (Thaulow et al., 1991). Their disc shape and negatively-charged surface glycoproteins are important in minimising platelet-platelet interaction. The production of platelets occurs in megakaryocytes, which are rare cells mainly found in bone marrow. Fully differentiated megakaryocytes form numerous cellular protrusions, or pro-platelets, which hold the majority of the cytoplasm. Platelets are believed to be formed at the end of these pro-platelets by granule component transfer from the megakaryocytes, up the pro-platelets, into the platelets. The platelets are then released into the blood stream, the mechanism has not been demonstrated but it is thought to be due to the action of proplatelet microtubules.

Platelet production is normally in the region of 1 x 10¹¹ per day, but synthesis can be increased to meet demand (Thaulow et al., 1991). The production of large numbers of platelets is required to maintain stasis due to the large turnover of platelets. The

approximate life-span of platelets is only 10 days and platelets are required in injury repair so, in these situations, there is an associated increase in platelet production. The spleen sequesters approximately one third of the circulating platelets and the liver removes the remaining non-functional platelets. Production and differentiation of megakaryocytes, and therefore production of platelets, is regulated by thrombopoietin, a glycoprotein produced by the liver, kidney, and bone marrow, although a number of cytokines (IL1, 6, and 11) are thought to play minor roles (Teramura et al., 1992, Wickenhauser et al., 1995).

Platelets contain a range of specialised organelles (Figure 2). One of these, the canalicular system is made up of a series of channels that are connected to each other and to the surface (White and Clawson, 1980, George, 2000, Flaumenhaft et al., 2005). The platelet cytosol contains contractile proteins, actin and myosin, which allow for shape change upon activation (Zucker-Franklin and Grusky, 1972). There are also specialised storage vesicles, α -granules and dense granules.

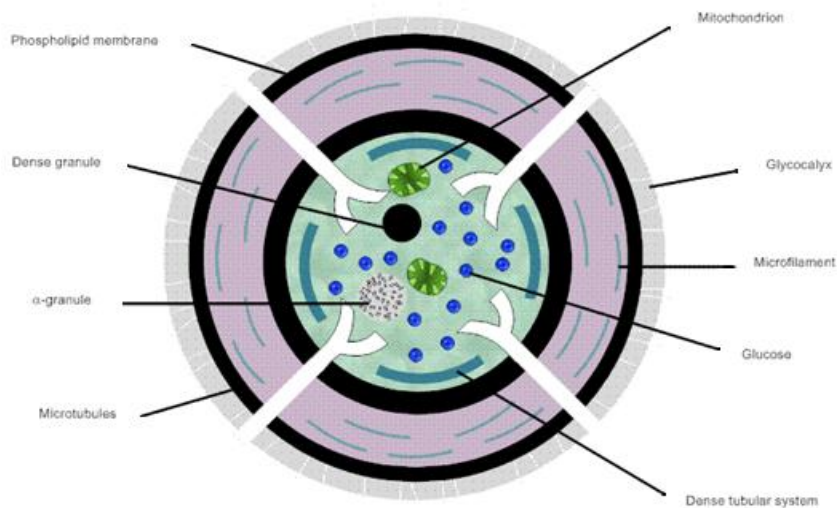
Upon stimulation by a number of compounds including ADP, adrenalin (epinephrine), collagen, and thrombin, contraction of cytoplasmic actin and myosin occurs. The platelets become irregular in shape, with pseudopodia protrusions. These allow for release of stored proteins (see Table 1 for an overview), via the canalicular system, from the α -granules (George, 2000, Zucker et al., 1979, Sweatt et al., 1985, Pengo et al., 1986, Lefebvre et al., 1993).

The dense granules contain stores of adenine nucleotides (ADP and ATP), serotonin (the vasoconstrictor 5-hydroxytryptamine), and histamine. There is also evidence to suggest that the inner membranes contain adhesion proteins, including glycoprotein Ib (GPIb) and glycoprotein IIb / IIIa (GPIIb/IIIa). Adenine nucleotides and adrenalin are linked with platelet aggregation. ADP is principally involved in platelet activation and therefore aggregation, while adrenalin may potentiate other platelet agonists. Serotonin is known to be involved in vasoconstriction. Serotonin and ATP are also known to be inflammatory mediators and as such may well be involved in repair processes as well as vascular disease (for specific references see Table 2).

Alpha granules contain a wide range of proteins, including adhesion proteins, such as GPIIb/IIIa, thrombospondin (TSP), and von Willebrand Factor (VWF); coagulation proteins such as factor V; plasma proteins such as albumin, fibrinogen, and immunoglobulin-G (IgG); growth factors such as platelet-derived growth factor (PDGF), vascular endothelial cell growth factor (VEGF) and transforming growth factor β (TGF- β); and some protease inhibitors. Gawaz (Gawaz, 2004) suggests that neutrophil-activating protein-2 (NAP-2) and plasminogen are found in α -granules, but more research is required to verify this. Some of these proteins, such as VWF, are synthesised by megakaryocytes prior to their fragmentation to form platelets. Others are acquired by platelets after they are formed, such as plasma fibrinogen, which is incorporated by

receptor-mediated endocytosis, and plasma proteins, which undergo fluid-phase pinocytosis (see Table 3 for specific references).

Figure 2: A Platelet



Both types of platelet granule contain calcium and magnesium, which associate with the coagulation factors in the coagulation cascade (Skaer et al., 1976). They also store proinflammatory cytokines and chemoattractants, the products of platelets themselves and internalized compounds (Ni et al., 2003, Boehlen and Clemetson, 2001). These mediators trigger platelet aggregation and can lead to coagulation and thrombosis.

Coagulation and thrombosis is the response to injury (and the current understanding of the processes involved are reviewed in (Monroe and Hoffman, 2006)). Blood vessel injury results in platelet activation via specific cell surface receptors, G-protein-coupled receptors, and the associated shape change, as detailed previously (Schafer et al., 2004). This shape change results in cell adhesion molecule upregulation. The cell adhesion molecules, and associated calcium ions released from the storage granules, bind coagulation factors VII, IX, X, and prothrombin (II) (Hoffman et al., 1995). These coagulation factors are present in their inactive form (proenzymes) in the blood. Activation occurs either by cleavage of part of the proenzyme or by conformational change to reveal the active site.

Coagulation factor X is the key factor in bringing about coagulation. This factor is required for the conversion of prothrombin to thrombin. For activation of factor X both platelets and tissue factor-bearing cells are required. Factor VII is bound to the tissue factor-bearing cells and when it is activated it will also activate factor X and factor IX. Factor Xa then binds with Va, which results in the cleaving of thrombin from prothrombin (Hoffman et al., 1995).

Activated factor VII from the tissue factor-bearing cells also interacts with platelet membranes. In so doing it can activate factor X. Platelets can also have factor Xa on their surface. Factor Xa leads to the conversion of prothrombin to thrombin (Esmon et al., 1974).

The production of thrombin requires calcium ions and phospholipids. Thrombin itself acts as an initiator for the production of more thrombin, albeit very slowly. This allows for control over thrombin formation. Cross linkage of the alpha chains of fibrin results in clot formation. This cross linkage is catalysed by coagulation factor XIIIa and calcium ions (Boyer et al., 1972).

Table 2: Platelet Dense Granule Content

Name	Function	Reference
Serotonin (5-HT)	Vasoconstrictor and inflammatory mediator	(Carty et al., 1981, Smith, 1996)
Adenine nucleotides (ATP, ADP)	Platelet activation and inflammatory mediator	(Meyers et al., 1982, Mori et al., 1984)
P-Selectin, GP Ib, GP IIb/IIIa	CAMs	(Youssefian et al., 1997)
Granulophysin (CD63)		(Israels et al., 1992)
Adrenalin & noradrenalin	Platelet aggregation and vasomotor tone	(Smith, 1996)
Calcium, phosphate & magnesium ions		(Skaer et al., 1976, Skaer et al., 1974)

(Key: 5-HT - 5-hydroxytryptamine; ATP - adenosine triphosphate; ADP - adenosine diphosphate; GP - glycoprotein; CAM - cell adhesion molecules)

Table 3: Platelet α -granule Proteins

Name	Function	References
GP IIb/IIIa	CAM for firm attachment	(Cramer et al., 1990)
TSP	CAM for platelet-neutrophil and platelet-matrix interactions	(Wencel-Drake et al., 1985)
VWF	CAM for platelet-collagen interaction	(Wencel-Drake et al., 1985)
Fibronectin, vitronectin, osteonectin, GP IV, GMP-33	CAM	(Berger et al., 1993, Wencel-Drake et al., 1985, Breton-Gorius et al., 1992, Metzelaar et al., 1992)
VWF:Ag II	Proprotein of VWF	(Scott and Montgomery, 1981)
Fibrinogen	Platelet adhesion via GPs and involvement in the clotting cascade	(Wencel-Drake et al., 1985)
Albumin	Plasma protein	(George and Saucerman, 1988)
Thromboglobulin, PDGF, VEGF and TGF- β	Cell proliferation and chemotaxis - smooth muscle cells, macrophages / monocytes & fibroblasts	(Assoian and Sporn, 1986, Salgado et al., 2001, Bowen-Pope et al., 1984, Kaplan et al., 1979)
IGF-1, ECGF, EGF, bFGF	Mitogenic growth factors	(Chan and Spencer, 1998, King and Buchwald, 1984)
PBP, CTAP-III	β -TG antigen molecules that are precursors for β -TG and PF-4	(Castor et al., 1983, Varma et al., 1982)
ENA-78, IgA, IgG, IgM, PF-4, β -TG	Immune response	(George et al., 1985, George and Saucerman, 1988, McLaren and Pepper, 1982)
PN-2	Inhibits protease and growth factor activity	(Van Nostrand et al., 1991)
PAI-1	Modulator of fibrinolytic activity	(Booth et al., 1988)
Platelet derived collagenase inhibitor	Modulates collagenase activity	(Cooper et al., 1985)
Protein S	Heparin cofactor - inactivate Factors Va and VIIa	(Schwarz et al., 1985)
α_2 -macroglobulin, α_2 -anti-plasmin, α_2 -anti-plasmin	Serine protease inhibitors	(Gogstad et al., 1983, Nachman and Harpel, 1976)
Multimerin	Binds factors VIII and V	(Hayward et al., 1998)
C1 inhibitor, factor V, factor VIII, HMK, HRGP	Coagulation	(Chesney et al., 1981, Leung et al., 1983, Schmaier et al., 1993, Schmaier et al., 1983) Yarerovi <i>et al.</i> 2003
RANTES & MIP	Cytokines	(Klinger et al., 1995)

(Key for Table 3: CAM - cell adhesion molecule; GP glycoprotein; TSP - thrombospondin; VWF - von Willebrand factor; VWF:Ag II - von Willebrand antigen II; PDGF - Platelet-derived growth factor ; VEGF - vascular endothelial cell growth factor; TGF- β - transforming growth factor β ; IGF-1 - insulin-like growth factor-1; ECGF - IGF-binding protein-3 endothelial cell growth factor; EGF - epidermal growth factor; bFGF - basic fibroblast growth factor; PBP - Platelet basic protein; CTAP-III - connective tissue activating protein-III; ENA-78 - Epithelial neutrophil-activating protein- 78; PF-4 - platelet factor-4; β -TG - β -thromboglobulin; PN-2 - Protease Nexin-2; PAI-1 - Plasminogen activator inhibitor-1; HMK High molecular weight kininogen; HRGP - Histadine-rich glycoprotein; RANTES - regulated upon activation, normal T cells expressed and secreted; MIP - macrophage inflammatory protein)

It is generally accepted that platelets play a key role in acute coronary events such as myocardial infarction, but there is some evidence that they are involved in initiation plaque formation. It has been proposed that platelets accelerate lesion formation by increasing monocyte migration to the intima (Dole et al., 2005, Schulz et al., 2007, Massberg et al., 2002). This has been corroborated by animal studies where platelet adhesion is impaired (Methia et al., 2001). Also, platelets bind, take up and transport oxLDL, resulting in activation of the platelet. These lipid laden activated platelets are then phagocytosed by monocytes resulting in foam cell formation (De Meyer et al., 2002).

2.4. Accelerated Atherosclerosis

It is widely accepted that atherosclerosis is an inflammatory state. There are many pro-inflammatory and inflammatory molecules involved in the development and progression of lesions and there is a link between bacterial infection and the severity of atherosclerosis and chronic vascular disease (Epstein et al., 2000, Zhu et al., 2000). Also, many inflammatory diseases are associated with accelerated atherosclerosis including diabetes, systematic lupus erythematosus (SLE), rheumatoid arthritis (RA), osteoarthritis (OA), human immunodeficiency virus (HIV), chronic obstructive pulmonary disease (COPD), and vasculitis including Wegener's granulomatosis (de Leeuw et al., 2005, Roman et al., 2003, Hannawi et al., 2007, Tabib et al., 2000, Pope et al., 2004, Jarvisalo et al., 2001, Conaghan et al., 2005). There are, however, some inflammatory conditions that have not been documented as being linked with accelerated rates of lesion formation including pelvic inflammatory disease, coeliac disease, and asthma. This suggests that there may be other factors that account for or contribute to accelerated atherosclerosis.

Accelerated atherosclerosis is associated with the above inflammatory disease but also with the following conditions also result in increased lesion formation: antiphospholipid syndrome, radiation, smoking, obesity and dialysis (Friedlander and Freymiller, 2003, Hoving et al., 2008, Pawlak et al., 2004, Lakka et al., 2001, Bolinder et al., 1997). It may be that these cause some sort of inflammatory response. Smoking, for instance, has been reported to cause inflammation as well as oxidative stress (Agarwal, 2005). It is possible however, that because some inflammatory diseases are not associated with accelerated lesion formation and that there are other non-inflammatory conditions associated with

accelerated atherosclerosis that other factors are involved. One theory that could link all these risk factors is activated platelets.

Activated platelets have been associated with RA (Endresen, 1989), diabetes (Tschoepe et al., 1991, Manduteanu et al., 1992, Shaw et al., 1967), COPD, SLE (Nagahama et al., 2001), smoking (Nair et al., 2001), antiphospholipid syndrome (Emmi et al., 1997), HIV (Holme et al., 1998), radiation (Gresner et al., 2005), obesity (Corsonello et al., 2003), dialysis (Tschoepe et al., 1991), Wegener's granulomatosis (de Leeuw et al., 2005), and chronic kidney disease (Stasko et al., 2007). These studies used various markers of platelet activation including, soluble P-selectin (sP-selectin), PMPs, Annexin V, cell adhesion molecules and cytokines.

A number of cell adhesion molecules have been reported to be up-regulated in diabetics including P-selectin and GPIIb/IIIa (Tschoepe et al., 1995, McDonagh et al., 2003). Increase in these adhesion molecules suggest that platelets become activated in diabetics. This is supported by reports of increased levels of platelet thromboxane (Lagarde et al., 1980). Evidence for an increase in plasma levels of granule proteins are, however, not as clear cut. The levels of β -thromboglobulin, for instance, have been shown to be increased in diabetics but, an increase was also reported in some non-diabetics (Burrows et al., 1978, Campbell et al., 1977). It is possible that an increase in β -thromboglobulin is associated with some predisposing conditions, such as obesity, and therefore many be present prior to diagnosis of diabetes.

2.5. Diabetes

Diabetes mellitus is a chronic metabolic disease that affects large numbers of people in the Western world and nearly every ethnic group. Evidence suggests that the disease has been around since ancient times, with symptoms detailed by the Egyptians and Romans (Patlak, 2002). In 2004 Wild and colleagues estimated that there were 171 million people over the age of 20 worldwide diagnosed as suffering from diabetes (Wild et al., 2004). This figure is unlikely to represent the total number of people with diabetes because this disease is inherently difficult to diagnose. Estimations, based on the current rate of increase, suggest that 344 million people globally will have diabetes by 2030 (Wild et al., 2004). As this figure is dependent on the prevalence of obesity remaining the same, it is probable that the actual number will be much higher due to the general increase in sedentary lifestyle and "fast food" culture. The already increased demands that diabetes has created on the NHS has been highlighted (Fitzsimons et al., 2002). These demands are on top of the increased expectations of patients for treatments that would previously only been available privately, and the increase in number of people prepared to seek compensation. Studies have suggested that diabetics cost the NHS more than twice as much as non-diabetics, with this cost increasing to 2 – 5 times as much when the cost of treating diabetic complications were included (Morsanutto et al., 2006).

Diabetes mellitus is a group of metabolic diseases that include type 1 diabetes mellitus and type 2 diabetes mellitus. They are both characterised by chronic hyperglycaemia (fasting glucose levels of greater than 7.0 mmol/L) resulting from reduced insulin secretion and/or sensitivity, but they differ in terms of clinical characteristics (such as age, weight and rate of onset), pathophysiology (for instance genetic factors), and epidemiology (WHO, 2006).

Type 1 accounts for approximately 10 % of patients with diabetes. This disease is characterised by autoimmune destruction of pancreatic insulin secreting β -cells. It is unknown what causes leukocytes to attack the β -cells, although genetic influences have been suggested and there is some evidence to suggest that environmental factors may be involved (Group, 2007, Gusdon et al., 2006). This destruction of β -cells causes decreased insulin secretion, which results in the inability of glucose to cross cell membranes and therefore poor regulation of blood glucose levels.

The pathogenesis of type 2 diabetes is also not fully understood at present, even though its high prevalence has led to a great deal of research in this area. It is thought that a number of factors play a role, such as dysregulated hepatic glucose production, impaired glucose tolerance, insulin resistance, and a slow decrease in pancreatic β -cell function (Basu et al., 2005, Festa et al., 2006). Many of these factors have been linked to obesity, a family history of Type 2 or gestational diabetes (which, although it is transient, it is similar to Type 2 except that the onset is linked to pregnancy), a sedentary lifestyle, and increasing longevity. In a small number of patients a genetic predisposition has been attributed, but poor lifestyle choices and other environmental factors are the primary factors in the development and progression of Type 2.

People with diabetes are more likely to suffer from cardiovascular disease than non-diabetics. It is the major cause of morbidity and mortality in patients with diabetes (Dale et al., 2007, Stamler et al., 1993, DCCT, 2005). A large proportion of premature deaths in diabetics are due to cardiovascular disease. There is a greater prevalence of cardiovascular disease in males than females. This gender gap, however, is reduced or eliminated when that female also has diabetes (Dale et al., 2007, Laing et al., 1999). Cardiovascular disease, therefore, is a serious complication of diabetes.

Diabetic vascular disease can be both micro- and macrovascular. Microvascular disease is associated with retinopathy, neuropathy, and nephropathy. These are thought to result from metabolic injury to the endothelium resulting from hyperglycaemia, changes in vascular reactive vasodilation, advanced glycation endproduct (AGE) formation, and endothelial injury (UKPDS, 1998, Kawano et al., 1999, Negrean et al., 2007). Macrovascular complications, such as atherosclerosis, do not show this clear link with hyperglycaemia, and therefore the underlying mechanisms are still being debated.

Diabetic accelerated atherosclerosis is thought to be due to hyperglycaemia because patients with poor glycaemic control are much more likely to suffer from cardiovascular

events associated with atherosclerosis (UKPDS, 1998). The processes involved in hyperglycaemia-induced atherosclerosis are not known. One theory suggests that AGEs alter endothelial cell function by causing upregulation and expression of cell adhesion molecules, pro-coagulatory and pro-inflammatory molecules, and transcription factors. This results in increased leukocyte and platelet adhesion to endothelium, and monocyte migration into the vessel wall. They are therefore likely to increase the formation of atheromas (Abordo et al., 1996, Kirstein et al., 1992, Schmidt et al., 1995).

AGEs are a heterogeneous group of compounds formed by autoxidation of Amadori products; a process known as the Maillard reaction. Amadori product is 1-amino-1-deoxyketose. This compound is produced when the carbonyl group of a reducing sugar attaches to the amino group of a protein. AGEs can also be formed from auto-oxidation of reducing sugars, by glycation intermediates, and by glycation of lipids. Once they have been formed, AGEs are almost completely irreversible.

The effect of AGEs can be three fold. Firstly, intercellular AGEs formation results in highly reactive intermediates that can directly alter protein function in cells that do not need insulin for glucose transport (such as endothelial cells). Secondly, extracellular AGEs can change matrix and cell interactions causing differences in permeability and tissue rigidity. And thirdly, AGE-receptor interactions alter gene expression by NF- κ B activation and by the production of ROS (Otero et al., 2001, Quehenberger et al., 2000, Schmidt et al., 1995).

AGEs are known to bind to cell surface receptors, such as scavenger receptors and RAGE (receptor for AGE). Binding of AGEs to RAGE, expressed on endothelial cells, results in activation of these cells and therefore increases endothelial permeability. This increase in permeability is thought to be the result of NF- κ B activation. The activation of this transcription factor results in increased gene expression of cytokines (such as IL6 and TNF- α), growth factors, and cell adhesion molecules (such as VCAM-1) (Morigi et al., 1998, Valencia et al., 2004).

AGEs also bind to monocytes, macrophages, and smooth muscle cells. This induces the production of inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-8 (IL-8), and tumour necrosis factor alpha (TNF α) (Pertynska-Marczewska et al., 2004). These cytokines are known to increase monocyte adhesion to endothelial cells, stimulate the proliferation of macrophages and smooth muscle cells.

Studies have been carried out to determine whether poor glycaemic control is the main cause of accelerated atherosclerosis (DCCT, 1993, Malmberg et al., 2005, Malmberg et al., 1999, UKPDS, 1998). Epidemiology studies indicate that there may be a correlation between glycaemic control and atherosclerosis. The Diabetes Mellitus Insulin Infusion in Acute Myocardial Infarction (DIGAMI) trial showed a decrease in mortality following aggressive treatment and control of hyperglycaemia but the DIGAMI 2 trial, which looked at different treatments, did not support the previous findings (although it did

indicate that glycaemic control was a marker of mortality following myocardial infarction) (Malmberg et al., 1999, Malmberg et al., 2005). Indeed, autopsy findings in people between the ages of 18 and 34 with poor glycaemic control indicated that they had an increased prevalence of more advanced plaques (Ledru et al., 2001). The DIGAMI, as well as the Diabetes Control and Complications (DCCT) Trial, indicated that there may be other factors, not just hyperglycaemia, involved in accelerated atherosclerosis (Malmberg et al., 1999, UKPDS, 1998).

Although atherosclerosis is believed to be an inflammatory disease, at present what causes this inflammation, or indeed whether this response is a key factor in the progression of accelerated atherosclerosis is unclear. Animal studies have indicated that endothelial cell injury in conjunction with hyperglycaemia results in lesion formation (Nishida et al., 1997, Ribau et al., 1998). Cell injury will result in an immune response and therefore it is possible that platelet adhesion will occur via exposed upregulated cell adhesion molecules, either directly to these adhesion molecules or via adherent monocytes.

Diabetic patients have impaired platelet function, as mentioned previously and therefore this may result in their adhesion to the endothelium. Diabetic platelets have also been shown to be glycated (Cohen et al., 1989). Glycation of platelet membrane proteins alters their function resulting in decreased fluidity (Watala et al., 1996). With both an increase in endothelial and platelet activation it is possible that these factors result in accelerated lesion formation in diabetics.

2.6. Overview of the Research Proposal

There is still no clear understanding of what initiates accelerated atherosclerosis in diabetics. Over the last 10 years, however, Gawaz and colleagues have published a number of papers suggesting that platelet adhesion may initiate lesion formation (Gawaz, 2006, Gawaz, 2004, Gawaz et al., 1998, Gawaz et al., 1997, Lindemann et al., 2007a, Siegel-Axel et al., 2008). Their rationale has been that blocking platelet adhesion results in reduced lesion formation and that platelets, unlike leukocytes express adhesion molecules that allow them to phagocytose modified lipids.

It is known that diabetes causes both endothelial and platelet dysfunction, so it is plausible that they could account for the accelerated rate seen in this disease. The mechanisms that result in dysfunction are, however, not clearly determined and whether platelets are a requirement for lesion formation to occur, or just play a minor role, needs further investigation. Therefore, *this research explores the hypothesis that the adhesion of activated platelets to the endothelium is required for atherosclerotic lesion formation; with specific reference to accelerated atherosclerosis seen in diabetics.*

In doing so, further understanding of the processes involved in diabetic associated accelerated atherogenesis. It is hoped that greater understanding of the processes

involved in accelerated lesion formation will allow for further targeted research to allow for treatments to reduce foam cell deposition. If platelets are found to be involved in the atherogenesis then further research could be done to investigate how they are involved, for instance, what kind of cell adhesion molecules are up-regulated, and drugs that specifically target this could be used in primary prevention as well as following acute manifestation of this condition, such as myocardial infarction. This would allow for extension in the length and quality of diabetic patient life, as well as significantly reducing the cost burden to the NHS of their treatment and care.

To do this, the initial aim of this research was to investigate whether platelets adhered directly to the endothelium in the early stages of atherosclerosis in diabetic-like conditions. These conditions were to be induced by treating cells with a combination of high concentrations of glucose and AGEs. Further investigations were to explore reports that platelet adhesion, in the form of platelet-leukocyte complexes, increase lesion formation and, if this is the case, whether this could partly be the cause of accelerated lesion formation seen in diabetics. In addition to the platelet studies, research into the role of endothelial dysfunction was carried out to investigate whether the conditions experienced in diabetics could exacerbate platelet adhesion via cell adhesion molecule, cytokine / chemokine expression, advanced glycated end-products and reactive oxygen production. To determine these, a combination of literature review, cell culture, and studies with isolated tissue were done. By doing this, it was proposed to determine whether endothelial dysfunction was induced by diabetic condition, whether this could allow for platelet adhesion, and whether this was the cause of accelerated atherosclerosis in diabetics.

3. Platelets Adhere to the Endothelium in Atherogenesis

3.1. Introduction

To determine whether adhesion of activated platelets to the endothelium is required for foam cell formation, it is first necessary to determine whether platelets can adhere to the endothelium in conditions that are known to result in lesion formation. If platelets do not adhere to the endothelium, either directly or indirectly, it is likely that, at best, they would only have a limited role in lesion formation.

Although Rotikansky suggested that blood component (platelet) deposition on the vessel wall caused lesion formation, until relatively recently it was thought that the endothelium was an inanimate monolayer that only provided a smooth surface that would deter thrombosis (Becker and Murphy, 1969, Jones, 1887, O'Nejll J, 1947, Samuels and Webster, 1952, Warren, 1963). It was believed that platelets adhered to the sub-endothelium only. Drs Sawyer and Srinivasan (1972) suggested that the endothelium acted as an anti-platelet 'policeman' by maintaining a negative electrochemical surface that repelled platelets. The underlying layers, however, were known to be positively charged and therefore would attract platelets. Endothelial denudation results in exposure of collagen and von Willebrand Factor (VWF), both of which were known to allow platelet adhesion.

However, as early as the 1960s there was some evidence to suggest that platelets might also adhere to the endothelium. Poole and colleagues (1963) found, by chance, adherent platelets in rabbits with very early thrombosis. Their studies of the small vessel in a rabbit ear chamber using electron microscopy indicated that platelets adhered to endothelial cells of damaged vessels. They reported that the platelets initially retained their discoid shape but following aggregation they became elongated and lost their granular appearance, suggesting activation and granule release. Leukocytes then adhered to the thrombus.

For thrombus formation to occur it would suggest that either the platelets were activated, the endothelium was activated / damaged, or that both were activated. Indeed, there was evidence to suggest any one of these possibilities could have resulted in platelet adhesion. Thrombin activated platelets have been reported to adhere to a monolayer of unactivated, undamaged, human endothelial cells (Czervionke *et al.* 1978). Cr-labelled platelets were found to adhere to cultured human umbilical cells (HUVECs) in the presence of thrombin. Adhesion increased with concentration but was significantly more when the endothelial cells were treated with aspirin. Treatment with relatively high concentrations of aspirin was likely to result in endothelial apoptosis but may also cause cell adhesion molecule up-regulation (Chen *et al.*, 2007a, Yoshida *et al.*, 1993). Endothelial activation, as a result of a reduction in PGI₂, following incubation with aspirin, is likely to account for this increase in platelet adhesion.

Non-activated platelets have also been reported to adhere to injured endothelium in male albino rats, cultured bovine cells, and pig specimens (Cotran and Majno, 1964, Booyse et al., 1975, Booyse et al., 1977). Booyse and colleagues suggested that activation of the endothelium by adrenalin, serotonin, sodium chloride, trypsin, heat treatment, and scraping all resulted in platelet adhesion (Booyse et al., 1975). This adhesion was reduced by treatment with platelet inhibitors (prostaglandin (PGE₁) and low concentrations of aspirin).

It wasn't until the 1980s, however, that platelet-endothelial interactions were thought to have a significant physiological role. This was mainly because there had not been any reports of platelet-endothelial interaction resulting in platelet activation and release of granule content. Granule release had only been reported as a result of platelet interactions with collagen or VWF present in the sub-endothelium (Mustard et al. 1977). Without release of granule components it was believed that platelet adhesion could have no significant role in dysfunctional states, including atherosclerosis.

Since then, it has become well established that platelets adhere to the sub-endothelium, the extracellular matrix, and to the endothelium, as seen in Table 4. Research has continued to investigate the finer points of platelet interaction with the vasculature, especially in association with disease states. From this research it was found that atherosclerotic lesion formation normally develops in areas where the endothelium is intact (Katsuda et al., 1992).

In autopsy studies of 15-35 year old humans, the majority of fixed vessel segments that contained either foam cells or fatty streaks had a thin layer of endothelial cells (Katsuda et al., 1992). This was determined by light microscope analysis. Immunocytochemistry studies, using ulex lectin and anti-von Willebrand factor antibodies confirmed that the endothelium was intact in all but a few cases.

Considerable research has, therefore, been carried out to investigate what types of adhesion molecules, platelets and endothelial cells express to allow them to interact. There are a number of cell adhesion molecules, that are either constitutively expressed or can be expressed upon activation, in both platelets and endothelial cells. Platelets and endothelial cells are known to express different types of adhesion molecules, including selectins, integrins, and members of the immunoglobulin family (see Table 5 and 6 for details).

Both endothelial cells and platelets also express a number of associated adhesion ligands that are known to be involved in cell-to-cell interactions. Most notable are a number of glycoproteins, such as fibrinogen, fibronectin, and VWF. These ligands are expressed and adhere to cell adhesion molecules. Like the cell adhesion molecules discussed previously, their expression can be increased upon activation.

From this research, it is clear to see that there are a number of adhesion molecules and ligands that play a significant role in platelet adhesion. These include P-selectin, VWF, VCAM-1, and GPIIb/IIIa. (The theoretical interactions are shown in Figure 3.) These were therefore highlighted as key to evaluating interaction in diabetic conditions to investigate whether platelet-endothelial interactions were required for lesion formation.

Table 4: Overview of Research Reporting Platelet-Endothelial Interactions

Cell line/Animal	Technique	Details	Reference
HUVECs & EAhy 926	Light microscope & flow cytometry	ADP activated platelets adhere to ECs	Author's own observations
BAECs	Plate reader under flow conditions	CRP-treated platelets adhered to ECs	(Yaron G et al., 2006)
HUVECs & SMCs	In situ fluorescent microscopy	Activated platelets adhered to ECs under flow conditions. Increased binding upon activation of both ECs & platelets	(Tull et al., 2006)
HUVECs	SEM	Platelet adhesion was dependent on glycocalyx levels	(Chen et al., 2007b)
<i>ApoE</i> ^{-/-} mouse	Video fluorescent microscopy	Platelet adhesion to the intact endothelium	(Massberg et al., 2002)
HUVECs	Fluorescent plate reader	Platelets adhered to VEGF activated ECs	(Verheul et al., 2000)
HUVECs	Flow cytometry	Platelets adhered to thrombin activated ECs	(Bombeli et al., 1998)
ECV-304	Flow cytometry	Activated platelets adhered to ECs. Increased adhesion when the ECs were also activated	(Gawaz et al., 1997)
VECs	Radiolabelled platelets & SEM	Diabetic platelets adhered to ECs	et al., 1992)
NZW	Radioactive platelets & scintillation counter	Platelet adhesion to the endothelium and to the subendothelium	(Tloti et al., 1991)
Rabbit thoracic aorta	Weight gain of the tissue	Platelet adhesion	(Korbut et al., 1990)
Wistar rats and guinea pigs	SEM of tissue	Platelet adhesion	(Maes et al., 1985)
BEACs	SEM & TEM with thrombin activated platelets	Platelet adhesion	(Trusal et al., 1984)
Pig artery	Microscope studies	Platelet adhesion was inhibited by aspirin or dipyridamole treatment	(Booyse et al., 1977, Silver et al., 1984)
HUVECs	Radioactive platelets	Thrombin activated platelets adhered to ECs	(Czervionke et al., 1978)
BAECs	Radioactive platelets	Platelet adhesion to ECs	(Booyse et al., 1975)
HAECs	Microscope studies	Platelets & ECs form intercellular attachments	(Rafelson et al., 1973)
Rabbit artery	Microscope studies	Platelet adhesion	(Poole et al., 1963)
Rat artery	Microscope studies	Platelet adhesion to gaps ECs	(Cotran and Majno, 1964)

(Key for Table 4: HUVECs - human umbilical vein endothelial cells; ADP - adenosine diphosphate; BAECs - bovine aorta endothelial cells; CRP - C-reactive protein; SMCs - smooth muscle cells; ECs - endothelial cells; SEM - scanning electron microscope; *ApoE*^{-/-} - apolipoprotein E deficient; VEGF - vascular endothelial growth factor; VECs - vascular endothelial cells; BAECs - bovine aortic endothelial cells; TEM - transmission electron microscopy; ECV-304 - Japanese spontaneously transformed cell line derived from HUVECs; NZW - New Zealand white mice; HAECs - human aortic artery cells)

(Key for Table 5: VWF - Von Willebrand factor; PSGL - P-selectin glycoprotein ligand; GP - glycoprotein; Fb - fibrinogen; Fn - fibronectin; Vn - vitronectin; TSP - thrombospondin; ADAM - a disintegrin and metalloproteinase; ICAM - intercellular adhesion molecule; LFA - lymphocyte function-associated antigen; PECAM - platelet-endothelial cell adhesion molecule; JAM - junctional adhesion molecule; VLA - very-late antigen)

Table 5: Expression of Cell Adhesion Molecules in Platelets

Name	Expression	Receptor for	Reference
P-selectin (CD62P)	On activation	PSGL-1, CD24	(Moore et al., 1995, Stenberg et al., 1985)
PSGL-1 (CD162)	Constitutively; increased upon activation	P-selectin	(Moore et al., 1995, Hsu-Lin et al., 1984, Frenette et al., 2000)
GP IIb/IIIa (CD41/61, $\alpha_{IIb}\beta_3$)	Constitutively; increased upon activation	Fb, Fn, VWF, Vn, TSP, ADAM15	(Bombeli et al., 1998, Langer et al., 2005, Nachman and Leung, 1982)
VLA-4 (CD49d/29, $\alpha_4\beta_1$)	On activation	VCAM-1, collagen, laminin & Fn	(Takada et al., 1987, Elices et al., 1990)
ICAM-2 (CD102)	Constitutively	LFA-1 ($\alpha_L\beta_2$), Mac-1 ($\alpha_M\beta_2$)	(Diacovo et al., 1996)
PECAM-1 (CD31)	Constitutively	PECAM-1 & $\alpha_v\beta_3$	(Albelda et al., 1991, Dhanjal et al., 2007)
JAM-A (F11R)	Constitutively	JAM-A	(Langer et al., 2005)
JAM-C	Constitutively	JAM-B, JAM-C, Mac-1 ($\alpha_M\beta_2$) & $\alpha_v\beta_2$	(Santoso et al., 2002)
GPIb (CD42a/b)	Constitutively	Thrombin, VWF, P-selectin, Mac-1, GPIb & TSP	(Nachman et al., 1979, Clemetson, 1983, Jurk et al., 2003, Romo et al., 1999, Takamatsu et al., 1986, Wang et al., 2005)
GP VI	Constitutively, increased upon activation	Collagen	(Clemetson et al., 1982)
GPIV (CD36)	On activation	Collagen & TSP	(Asch et al., 1987, Tandon et al., 1989)
GPV (CD42d)		VWF, thrombin	(Calverley et al., 1995, Dong et al., 1997)
$\alpha_v\beta_3$ (CD51/61)	On activation	Osteopontin, Fb, Fn, Vn, TSP, VWF, LI & PECAM-1	(Bennett et al., 1997, Columbo and Bochner, 2001, Gawaz, 2004)
GPIa/IIa ($\alpha_2\beta_1$, CD49b/29, VLA-2)	Constitutively	Collagen and laminin	(Elices and Hemler, 1989, Saelman et al., 1994)
VLA-5 (CD49e/29, $\alpha_5\beta_1$)	Constitutively	CD40 ligand, Fb, Fn, & LI	(Hemler et al., 1988, Podolnikova et al., 2003, Ruppert et al., 1995)
VLA-6 (CD49f, $\alpha_6\beta_4$)		Laminin	(Hemler et al., 1988, Sonnenberg et al., 1988)

Table 6: Expression of Cell Adhesion Molecules on Endothelial Cells

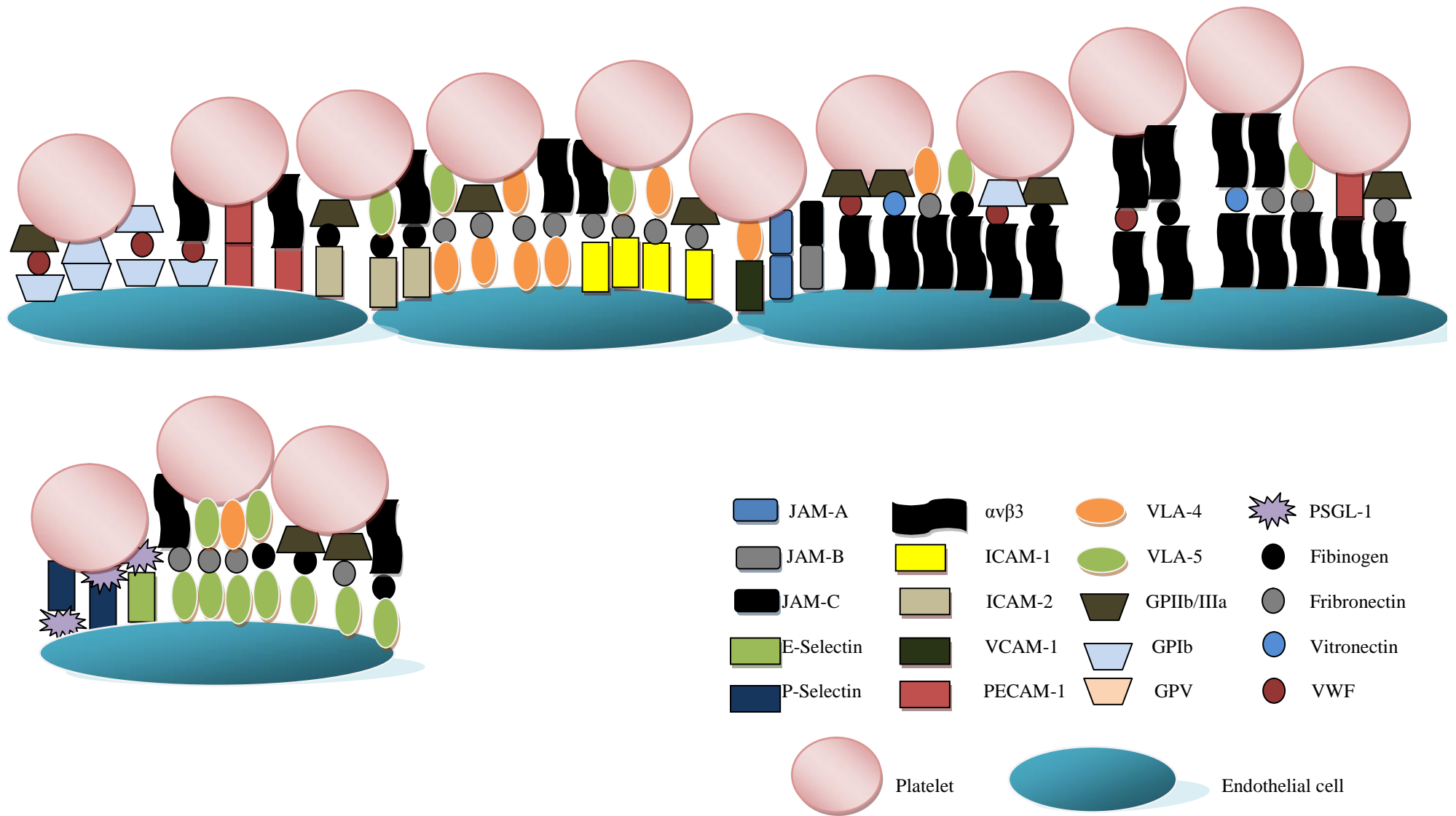
Name	Expression	Receptor for	Reference
P-Selectin (CD62P)	On activation	PSGL-1, CD24	(Chen et al., 2007b, Johnston et al., 1989)
E-Selectin (CD62E)	On activation	PSGL-1, ESL-1, L-Selectin	(Wild et al., 2001, Bevilacqua et al., 1987)
PSGL-1 (CD162)	Constitutively; increased on activation	P-Selectin	(Chen et al., 2007b, da Costa Martins et al., 2007)
GPIIb/IIIa (CD49b/29, $\alpha_2\beta_1$, VLA-2)	Constitutively	Collagen	(Zutter and Santoro, 1990)
VLA-4 (CD49d/29, $\alpha_4\beta_1$)	On activation	VCAM-1, Fn	(Elices et al., 1990, Garmy-Susini et al., 2005)
$\alpha_v\beta_5$ (CD51)	On activation	Collagen, Fn	(Pedchenko et al., 2004, Walton et al., 2000)
ICAM-1 (CD54)	Constitutively	Fn, LFA-1, Mac-1, $\alpha_x\beta_2$	(Bombeli et al., 1998, Abbassi et al., 1993, Steinhoff et al., 1993, D'Souza et al., 1996, Dustin and Springer, 1988)
ICAM-2 (CD102)	Constitutively	Fb, LFA-1, Mac-1	(de Fougerolles et al., 1991)
VCAM-1 (CD106)	Constitutively	VLA-4, $\alpha_4\beta_7$, $\alpha_D\beta_3$	(Van der Vieren et al., 1999, Dobrina et al., 1991, Newham et al., 1997)
PECAM-1 (CD31)	Constitutively	PECAM-1, $\alpha_v\beta_3$	(Albelda et al., 1990, Sun et al., 1996, Piali et al., 1995)
JAM-A	Constitutively	JAM-A, LFA-1	(Aurrand-Lions et al., 2001, Ozaki et al., 1999)
JAM-B	Constitutively	JAM-B, JAM-C, VLA-4	(Arrate et al., 2001, Aurrand-Lions et al., 2001, Cunningham et al., 2000, Cunningham et al., 2002, Ludwig et al., 2005)
LOX-1	On activation	Include modified lipoproteins & activated platelets	(Moriwaki et al., 1998)
GPIb (CD42)	On activation	VWF, Mac-1, TSP, thrombin, GPIb, P-Selectin	(Bombeli et al., 1998, Clemetson, 1983, Jurk et al., 2003, Takamatsu et al., 1986, Wang et al., 2005, Romo et al., 1999, Jones et al., 1981)
$\alpha_v\beta_3$ (CD36)	On activation	Osteopontin, Fn, Fb, Vn, TSP, VWF, PECAM-1	(Tandon et al., 1989, Asch et al., 1987)
GPIV (CD36)	On activation	Collagen, TSP	(Asch et al., 1987, Tandon et al., 1989)

Table 6 Continued: Expression of Cell Adhesion Molecules on Endothelial Cells

Name	Expression	Receptor for	Reference
VLA-6 (CD49f/29, $\alpha_6\beta_1$)	Constitutively	Laminin	(Leu et al., 2002)
$\alpha_6\beta_4$ (CD49f/104)	Constitutively	Laminin	(Sepp et al., 1995, Sonnenberg et al., 1988)
VLA-5 (CD49e/29, $\alpha_5\beta_1$)	Constitutively; increased on activation	Fb & fn	(Suehiro et al., 1997, Collo and Pepper, 1999)
CD147	On activation	MMP-1	(Seulberger et al., 1992, Guo et al., 2000)
N-cadherin	On activation	N-cadherin	(Salomon et al., 1992)
VE-cadherin (CD144)	Constitutively	VE-cadherin	(Lampugnani et al., 1995)
MUC18 (CD146)	On activation	Not known	(Bardin et al., 2001, Sers et al., 1994)
Syndecan-1 (CD138)	On activation	Laminin	(Hoffman et al., 1998, Kainulainen et al., 1996)
CD39	Constitutively	CD39	(Kansas et al., 1991, Wang and Guidotti, 1998)
PCLP-1	Constitutively	L-selectin	(Sasseti et al., 1998, Kershaw et al., 1997)

(Key for Table 6: PSGL-1 – P-Selectin glycoprotein ligand; ESL – E-Selectin ligand; VLA – Very late antigen; VCAM – vascular cell adhesion molecule; Fn – Fibronectin, ICAM – Intracellular adhesion molecule; LFA - lymphocyte function-associated antigen; PECAM – Platelet-endothelial cell adhesion molecule, JAM – junction adhesion molecule; LOX - Lectin-like oxidized low-density lipoprotein receptor; Fb – Fibrinogen, TSP – Thrombospondin, VWF, Von Willibrand factor, MMP - matrix metalloproteinases; N-cadherin - neural cadherin; VE-cadherin - vascular endothelial cadherin; PCLP - podocalyxin-like protein)

Figure 3: Theoretical Representation of Interactions between Platelet and Endothelial Cells.



3.2. My Proposed Research

There has been considerable research into platelet activation but this has failed to establish whether platelets are involved in the initial stages of atherosclerosis. In order to establish this, I proposed to investigate whether diabetic conditions resulted in platelet adhesion to cultured endothelial cells. Initially, studies were to involve mimicking diabetic conditions by treating cultured monolayers of commercial endothelial cells with either high concentrations of glucose or AGEs, or a combination of both. Adhesion was to be determined by fluorescently labelling the platelets and using a fluorescent microscope or a flow cytometer. Platelet adhesion would then be compared against control conditions to determine whether any increase in adhesion was statistically significant. By doing so, it was hoped that this would give an insight into whether platelet adhesion could be involved in accelerated lesion formation in diabetics.

There has already been research published that has shown diabetes causes changes to platelets, including activation and increased adhesion to the endothelium (Manduteanu et al., 1992, Nievelstein et al., 1991, Wautier et al., 1981). Obviously, changes to platelets are only part of the possible effect of diabetes on interactions between platelets and the endothelium. There appears to be, however, no research into the effects of non-activated (or non-diabetic) platelet adhesion to diabetic endothelium. Therefore, this leaves a gap in knowledge that may hold the key into understanding the processes involved.

High glucose and AGE concentrations were used for these studies because diabetes is associated with increased levels of blood glucose and glycation of proteins (both of which have been reported to increase leukocyte adhesion by increasing cell adhesion molecule expression (Morigi et al., 1998, Altannavch et al., 2004)). It is therefore possible that this would result in increased platelet adhesion, although there appears to be no published research investigating in vitro platelet-endothelial interactions under diabetic conditions.

Research using AGEs, however, appear to give conflicting results (Otero et al., 2001, Morigi et al., 1998, Gilcrease and Hoover, 1991, Altannavch et al., 2004). Various studies have reported that AGEs increase leukocyte adhesion, Gilcrease & Hoover, however, found that the apparent increase in monocyte adhesion seen in vitro was not directly related to the increase in AGE formation but as a result of increased NaCl concentrations. Another paper found no significant increase in monocyte adhesion under static conditions (Kim et al., 1994). Therefore, as part of this research I proposed to look at whether the type of protein used for glycation purposes affected platelet adhesion. Most AGE studies have restricted their research to using albumin (generally bovine serum albumin). As such, by comparing platelet adhesion following endothelial incubation with both albumin and haemoglobin I hope to get a greater understanding.

Further to these studies, I wanted to establish whether the length of time the protein was exposed to high concentrations of glucose, and therefore the extent of glycation, could also have an impact on platelet adhesion. To enable this, platelet adhesion studies were done with 2 – 12 weeks (in 2 week intervals) glycated proteins.

3.2.1. Peptide Synthesis of Part of Albumin

To complement this study, a sequence of albumin that did not contain the majority of known drug binding sites (between residues 124 and 585), was chosen for synthesis and then glycation (Fehske et al., 1981). It was hoped that by omitting the majority of known drug binding sites it would reduce the likelihood of unspecific binding. The sequence, however, contained amino acids that were known to be involved in the glycation process, such as arginine, histidine and lysine. It was also taken into consideration that lysine and glutamate catalyse the glycation of nearby lysines (Johansen et al., 2006, Coussons et al., 1997).

Synthesis of the selected sequence was carried out using an N- α -Fmoc strategy, based on the Merrifield Technique (Merrifield, 1969). Rink amide resin was used to build a 12 amino acid sequence (His-Arg-Phe-Lys-Asp-Leu-Gly-Glu-Glu-Asn-Phe-Lys). Following synthesis, the peptide was purified using High Performance Chromatography (HPLC) prior to glycation with *D*-glucose.

3.2.2. Glycation

The peptide, haemoglobin and bovine serum album were glycated with *D*-glucose using a slight adaption to the technique detailed by Morigi and colleagues (Morigi et al., 1998). Various purities of albumin were glycated. These included greater than 96%, 98% and 99%. The rate of glycation, for both the peptide and the proteins, were recorded, at 2 week intervals, using MALDI-TOF (Matrix assisted laser desorption ionization - time of flight) and fluorescent spectrophotometry. Finally, any unincorporated glucose was removed from the glycated peptide and proteins using dialysis before quantifying the concentration using a protein assay kit.

3.2.3. Platelet Adhesion Assay – Flow Cytometry

The various types of glycated proteins / peptide were added to the medium of the cultured endothelial cells, with or without glucose, to determine whether they resulted in an increase in platelet adhesion. Flow cytometry was chosen for detection of endothelial bound platelets because it allows for investigating the interaction of these cells without the possibility that the extracellular matrix or other components resulted in platelet interactions.

A method published by Bombeli and colleagues was chosen for these studies (Bombeli et al., 1998). They used flow cytometry to investigate the cell adhesion molecules involved in platelet-endothelial interactions and therefore the method was believed to be ideal for platelet adhesion studies. Slight variations were made because it was found that their use of vigorous pipetting did not successfully detach endothelial cells from the culture flask. The use of a cell scraper created a lot of cellular debris and therefore trypsin detachment was preferred. (This was also the standard method recommended by the cell supplier.) The procedure also used 80% ethanol to fix the endothelial cells. This resulted in fragmentation. As such, various other methods were investigated, including concentrations of ethanol or methanol/acetone but it was found that a flow cytometry cell fix solution (saline solution containing 4% w/v paraformaldehyde) resulted in the minimum amount of fragmentation and cellular debris.

To visualize platelet-endothelial conjugates, endothelial cells were stained using propidium iodide (PI), an intercalating agent that stains DNA. This resulted in the endothelial cells emitting in the 562-588 nm spectrum, and therefore they were detected by scatter from the FL2 filter. Platelets were stained with acetomethoxy calcein. Calcein is a fluorescent dye, emitting at 515nm, which binds to intracellular calcium in such a way that there is no transfer between cells. Light scatter from the FL1 filter allowed for platelet detection and platelet-endothelial complexes were both FL1 and FL2 positive.

There are a number of ways to investigate cell-cell adhesion, including flow cytometry, immunocytochemistry, and microscope studies. Flow cytometry was used to reduce the potential experimental variables associated with using monolayers. It is known that in vitro studies using endothelial monolayers can give misleading results. Chen and colleagues found that the amount of platelet adhesion to human umbilical endothelial cells (HUVECs) cultured on hydrogel scaffolds was dependent on the type of gel that was used and this was related to the amount of glycocalyx produced by the cells (Chen et al., 2007b). Glycocalyx covers the luminal surface of endothelial cells and is known to have a role in their alignment under shear stress (Yao et al., 2007).

Cell preparation, therefore, can have a large impact on platelet adhesion. Indeed, it has also been shown to affect interaction with the endothelial extracellular matrix (Aznar-Salatti et al., 1991). This suggests that investigations into platelet interactions can give misleading results. It was noted, for instance, that 13-hydroxy octadecadienoic acid (13-HODE) concentration and distribution pattern vary based on how the cells were prepared. 13-HODE is a lipoxigenase pathway intermediate that is known to be an inhibitor of platelet-platelet interaction (Coene et al., 1986). It has been reported that this compound inhibited platelet adhesion to both the endothelial monolayer and to the extracellular matrix. There

was a greater inhibitory effect on the endothelium than on the extracellular matrix (Tloti et al., 1991).

To minimize variation in cell preparation on platelet-endothelial interactions, HUVECs and all cell media were to be purchased from a reputable supplier (Cambrex). The cells were to be stored and cultured as per the supplier's instructions and all experiments were to be performed using cells between passages 4-6. Negative controls consisted of endothelial cells cultured in normal conditions, with 100 µg/ml of non-glycated protein or unactivated platelets, while adenosine diphosphate (ADP) activated platelets were used as a positive control. These were used to compare the affect of incubation of the endothelial cells with high glucose (11mM and 22 mM) and/or AGEs (100 µg/mL) for 24 and 48 hours on platelet adhesion. These times were chosen so that comparison could be made with other published results to determine whether the type of protein could account for discrepancies. All experiments were carried out in triplicate (with 3 samples for each control and treatment) and the whole experiment was repeated 3 times to check for reproducibility and allow for statistical analysis.

3.2.4. Platelet Adhesion Assay – Immunocytochemistry

Obviously, although flow cytometry allows for investigation into platelet and endothelial interactions and rules out the involvement of the extracellular matrix, there are a number of limitations to this technique. This includes the fact that endothelial cells are not normally found in isolation. They are present as a monolayer and are elongated rather than spherical. This may result in endothelial cell adhesion molecules, such as junction adhesion molecules (JAM), being available when they would otherwise not be. For analysis, the endothelial cells also need to be removed from the culture flask. This requires either cell scraping or enzyme treatment. This leads to cell disruption and an increase in potential for cellular debris.

Immunocytochemistry was used to complement the flow cytometry results, to give an insight into the role of the endothelial monolayer, as opposed to endothelial cells in suspension. Both fluorescent and confocal microscope analysis were run in parallel. Endothelial cell culture was performed in 6-well plates containing sterile coverslips. (In situ coverslips were used, as opposed to growing the cells to confluence and then transferring them to coverslips, to reduce the amount of disturbance required for the experiments. Once the cells were between 75 and 85 % confluent (depending on whether they were being incubated for 24 or 48 hours) any treatments were initiated (as mentioned above). Cell fixing procedures were investigated to minimize cell disturbance, including various concentrations of methanol, methanol/acetone, acetone, paraformaldehyde and ethanol. From some initial investigations, many of these fixation methods appeared to result in protein denaturing and endothelial cell

detachment. The best results were obtained using 70% ethanol and 4% paraformaldehyde fixation. I proposed to label VCAM-1 using an anti-VCAM-1 antibody and then a Texas red conjugated secondary antibody. The endothelial nucleus was to be stained using 4',6-diamidino-2-phenylindole (DAPI) and platelets labelled with FITC-CD42b antibodies. A mounting medium would also be used to prevent loss of fluorescence and bleaching of fluorescein isothiocyanate (FITC).

3.2.5. Dynamic Flow

Neither of the above procedures ruled out the effect of sub-endothelial interactions that are likely to take place in vivo, the effect of dynamic flow within the vessel or indeed, the fixation and staining methods needed for these studies. It was therefore proposed to run time-lapse studies using a flow chamber, both with endothelial monolayers and with vascular biopsy specimens from diabetic patients.

The use of diabetic tissues would obviously have some disadvantages in that the tissue would have been taken from diseased vessels. The process of removing the disease tissue would however include a margin of non-diseased tissue (to make sure that all the diseased area was removed). This non-diseased tissue would have been subjected to diabetic conditions and therefore would give a good indication of whether platelet adhesion was increased. Obviously, the biopsy procedure may well have caused some stress to the sample but, it would still provide a comparison for cell culture studies.

The tissue would be placed or the cells would be cultured on a coverslip prior to placing into a parallel-plate flow chamber with perfusion system, as described by Lawrence and colleagues (Lawrence et al., 1987). Platelet-rich-plasma, from a reservoir at 37°C, would then be circulated, by a peristaltic pump, at physiologically normal (0.9 dyne/cm²) and high shear rates (1.5 & 3 dyne/cm²) to investigate the effect of dynamic flow conditions on platelet adhesion to the endothelium. Platelet adhesion would be investigated under normal conditions, with ADP activated platelets for the controls and then with high glucose and or AGE incubated endothelial cells. Images were to be obtained by using a video recorder connected to a light microscope.

3.2.6. Research To Date

3.2.6.1. Platelet Adhesion to the Endothelium

Various investigations have looked into the adhesion molecules involved in platelet-endothelial interactions. Bombeli and colleagues used monoclonal antibodies and flow cytometry to determine that platelets adhered to endothelial cells (Bombeli et al., 1998). They found that platelets adhered to

apoptotic HUVECs, partly via β_1 integrins ($\alpha_2\beta_1$ (GPIa/IIa), $\alpha_5\beta_1$ (VLA-5), $\alpha_6\beta_1$ (VLA-6)) and by GPIIb/IIIa. Blocking of all three platelet β_1 integrins only resulted in a 20 % reduction in platelet adhesion, while blocking GPIIb/IIIa reduced adhesion by approximately 50 %. Platelet adhesion was also found to be significantly reduced by blocking endothelial ICAM-1, $\alpha_v\beta_3$, GPIb. Blocking of endothelial E-Selectin and VCAM-1, platelet GPIb and GPIV, and both endothelial and platelet PECAM-1 and P-Selectin showed no significant decrease in adhesion.

This research highlighted that results differ based on what monoclonal antibody is used. There were a number of different antibodies used for a specific cell adhesion molecule. The majority selected for this investigation did not vary significantly but there were large differences for anti- $\alpha_v\beta_3$. This is probably because one was blocking and the other non-blocking. The blocking antibodies used also only inhibited specific types of binding. For instance, ICAM-1 monoclonal antibody P6.5 blocks the β_2 integrin-binding site while 2D5 blocks the fibrinogen-binding site.

Others have also suggested that GPIIb/IIIa was responsible for firm, but not for transient adhesion (Massberg et al., 2002, Gawaz et al., 1997, Verheul et al., 2000). They also reported that platelets deficient in GPIIb/IIIa did not firmly adhere to activated endothelial cells. However, blocking $\alpha_v\beta_3$ or GPIb α resulted in reduced platelet adhesion, suggesting that platelet adhesion via GPIIb/IIIa was either GPIb or $\alpha_v\beta_3$ -dependent.

Further studies have confirmed that GPIb is involved in platelet adhesion to activated endothelium using HUVECs or mice (Andre et al., 2000, Etingin et al., 1993). Blocking VWF, a ligand for GPIb, was reported to reduce platelet adhesion to virally infected cells by 90 %. Another investigation using HUVECs found that blocking GPIb had no effect on platelet adhesion (Verheul et al., 2000). These discrepancies may result from culture differences. Most of these studies are in vitro and have centred on the use of cultured cells. The use of cells grown as monolayers has its own drawbacks, including batch-to-batch variation, differences in cell adhesion molecule expression with increasing passage number, and due to origin; therefore the results may not reflect physiological situations. Also, by using blocking studies variability occurs from differences in the antibody binding affinity and cell adhesion molecule expression levels. By looking at knock-out models there is the potential to carry out in vivo experiments, which should give a more accurate representation of physiological conditions.

Animal studies have been performed to look at platelet adhesion to the endothelium (Korbut et al., 1990, Pettersson and Bjork, 1992, Tloti et al., 1991). Korbut and colleagues (Korbut et al., 1990) detailed a model for

investigating adhesion under flow conditions. Rabbit aortic segments were attached to apparatus that allowed for weight measurements to be taken to determine thrombus formation. During their investigation they observed that increased weight was associated with thrombus formation. Microscope analysis determined platelet adhesion to the intact endothelium.

Radioactively-labelled platelets have also been used to analyse platelet adhesion in rabbits (Pettersson and Bjork, 1992). The rabbit vein was cannulated to allow for administration of the radioactively-labelled platelets into healthy rabbits. They observed platelet adhesion to the vessel wall. (Deposition of platelets was greater at regions of vessel branching. This is where lesion formation is reported to be most likely to occur (Tanganelli et al., 1993).) However, these findings were not confirmed at slaughter and therefore could have been the result of transient attachment or platelet aggregation. If transient attachment occurs, cross-talk (communication potentially involving activation or transfer of cytokines) between the platelets and endothelial cells is possible. Detection of this, either in vivo or in autopsy findings, would potentially be problematic because of the transient nature.

Platelet adherence to sheep pulmonary artery endothelial cells has also been reported (Tloti et al., 1991). Platelet adherence increased approximately threefold then the platelets were activated by thrombin. Co-incubation of activated platelets and activated endothelial cells resulted in nearly a fivefold increase in platelet adhesion when compared to non-treated cells. This would indicate that platelet adhesion is significantly increased when both the endothelium and the platelets are activated.

It has been reported that mice lacking endothelial P-selectin exhibited decreased platelet adhesion when compared to wild type mice (Frenette et al., 1998, Frenette et al., 1995). When P-selectin deficient platelets were introduced adhesion still occurred, suggesting that endothelial, but not platelet, P-selectin is involved in platelet-endothelial interactions. These findings contradict other investigations (Andre et al., 2000, Bombeli et al., 1998, Verheul et al., 2000). This may be because the in vivo studies used intravital microscopy in the mouse tail vein and mesenteric venules, while the in vitro investigations used umbilical vein endothelial cells. It is also possible that the origin of the endothelium, including species and vessel type, could result in cell variance, which includes cell adhesion molecule expression (Kuzu et al., 1993). Obviously, although in vivo animal experiments are more likely to mimic the conditions that are available naturally, in animals, the findings may not give an accurate picture of what happens in humans. It is possible that they may also be specific to those species only. Also, the methods used are often invasive and therefore many unknown variables could be introduced including activation of both endothelial cells and platelets.

It is clear to see that there are some discrepancies in the literature as to what adhesion molecules are involved. It does, however, indicate that platelets do indeed adhere to the endothelium. This interaction does not rely on one set of adhesion molecules. It is likely that many are involved, with potentially physiological conditions determining certain interactions. These studies, however, do not determine whether platelet adhesion is involved in the initial stages of atherosclerosis. Other research has been done that may give insight into whether platelet-endothelial interactions initiate lesion formation. These include studies on lesion formation, blocking of platelet adhesion, and whether platelet abnormalities reduce or stop atherogenesis.

3.2.6.2. Platelet Adhesion in Atherogenesis

Histological and immunocytochemical investigations have been carried out to investigate the stages of atherosclerotic lesion formation and provide classification (Gown et al., 1986, Katsuda et al., 1992, Stary et al., 1994, Stary, 2000, Stary et al., 1995). During these studies platelet adhesion was not associated with the initial stages of atherosclerosis, only the later stages where the fibrous plaque becomes brittle and can fissure – exposing collagen and other thrombogenic components, and therefore allowing for platelet adhesion.

The initial stages are usually associated with leukocyte and not platelet adhesion. Indeed, Walker and colleagues (Walker et al., 1986) studied leukocyte and platelet adhesion to rabbit endothelium. They reported leukocyte adhesion to the initial stages of atherosclerosis (type 1 and type 2 lesions; see table 1 for details), but no platelet interactions. There was no mention of platelet adhesion. The fixation and preparations used for imaging the thoracic artery were unlikely to have resulted in detachment of adhered platelets but would certainly have removed transiently attached cells. It is therefore unlikely in these rabbits that firm adhesion platelets occurred in the initial stages of atherosclerosis.

In humans, there is a distinctive lack of publications reporting platelet adhesion to the endothelium. There are, however, microscope investigations into lesion formation that show leukocyte adhesion (Emeson and Robertson, 1988, Millonig et al., 2002, Stary, 1990, Stary and Malinow, 1982). An overview of these papers can be found in Table 7. Many of these studies have specifically looked for leukocyte adhesion. For instance, the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study used antibodies to investigate leukocyte interactions (Millonig et al., 2002). Sections of aortic tissue from people aged 17 to 34, who had no clinical signs of cardiovascular disease, were labelled with monoclonal antibodies for T-lymphocytes, interleukin-2 receptors, macrophages, dendritic cells, mast cells, natural killer

cells, major histocompatibility complex class II-expressing cells (macrophages, dendritic cells and B-cells), and B-cells. As such, any adherent platelets would not be detected.

The lack of evidence for adherent platelets may well be telling in its own right however, it could be a result of the methodology. There is also, no mention of adherent platelets, unless in relation to advanced lesions. These investigations involve fixing, blocking, with their various washing stages, and immunocytochemistry imaging. These processes are likely to eliminate any transiently attached platelets, but may also result in detachment of those that are more firmly adhered. This is because the studies were centred on leukocyte adhesion and therefore platelet monoclonal antibodies were not used. Platelets would therefore not be picked up by fluorescent microscopy.

Spurlock and Chandler (Spurlock and Chandler, 1987), however, reported adhesion of isolated platelets or micro-thrombi to arterial endothelium in a small percentage of people, aged 15-34, who died from sudden trauma. Analysis was carried out using a scanning electron microscope (SEM). The SEM allowed for rapid examination of large sections of intima. This observation does not directly suggest that platelet adhesion occurs prior to lesion formation, but it is known that lesions occur in childhood, so there may be a link. Indeed, platelet adhesion has been reported to occur prior to leukocyte adhesion and before lesion formation in mice (Massberg et al., 2002). Platelet adhesion was observed *ex vivo* at sites where later lesions occurred. Increasing age corresponded with increased platelet adhesion in the atherosclerotic prone apolipoprotein E deficient (Apo E^{-/-}), but not in the wild type mice. Blockade of GPIb α and GPIIb/IIIa resulted in reduced leukocyte accumulation in the intima and also decreased lesion formation. This would suggest that platelets are required for leukocyte adhesion because GPIIb/IIIa is expressed constitutively by platelets and allows for leukocyte adhesion via Mac-1. Lack of Apo E results in increased fat deposition and therefore lesion formation, making these mice a very useful tool for studies into atherosclerosis, however, the results may not resemble human physiology. It is possible that the inability to catabolise lipoproteins causes specific endothelium injury resulting in platelet adhesion, which is not seen in endothelial dysfunction related to hyperglycaemia, for instance.

Another report found that platelets tethered, rolled, and arrested on early lesions in this atherosclerosis mouse model (Huo et al., 2003). Adhesion was mainly for short periods of time. It was thought that this transient adhesion allowed for transfer of platelet mediators, such as regulated upon activation normal T-cell expressed and secreted (RANTES) and platelet factor 4 (PF-4). Platelet adhesion, however, was reported to be rarely in isolation. Platelets mainly adhered to monocytes, via platelet P-selectin, and promoted monocyte

adhesion to the endothelium. These findings partially concur with those of Massberg and colleagues (Massberg et al., 2002), but because this investigation focused on platelet and leukocyte adhesion prior to lesion formation it is not possible to determine whether platelet adhesion initiates lesion formation. There was also no definition for 'early lesion'. Mean lesion area coverage was determined at eighteen weeks on an atherogenic diet. However, it is clear that platelets are involved prior to advanced lesion formation in Apo E^{-/-} mice.

Platelet adhesion prior to noticeable lesion formation was also found in rabbits (Theilmeier et al., 2002, De Meyer et al., 1999). It was found that platelet adhesion occurred after three months of the rabbits being fed on an atherogenic diet. Although the rabbits exhibited mild hypercholesterolaemia, no fatty streaks were present until twelve months of the diet. Platelet adhesion was seen at sites near segmented arteries, where lesions would be expected. Platelets were not found adhered to other areas of the arteries. The use of ex vivo and in vitro experiments determined that platelet adhesion was due to increased endothelial VWF (-GPIb) and platelet P-selectin. This is partly supported by other studies that have reported that there was a significant increase in platelet adhesion at the carotid bifurcation compared to the proximal carotid artery in mice (Massberg et al., 2002).

These reports suggest that platelet-endothelial interactions occur prior to lesion formation. Obviously, this cannot be stated as fact because, using these methods, it is not possible to see the time lapse of events. If it was, then evidence showing that following on from platelet adhesion monocytes interacted with the endothelium and differentiated into macrophages and then developed into foam cells and lesions would give greater clarity. To do this kind of research, however, would result in disturbance to the endothelium and therefore damage would occur. As such, this in its own right might result in platelet and monocyte interactions for wound healing and infection control. It would therefore be impossible to determine the sequence of events in accelerated atherosclerosis in diabetes.

Although there is some evidence to suggest that platelets adhere to the endothelium prior to lesion formation this is more circumstantial rather than direct. Perhaps it is telling that there is very little evidence to suggest that platelets adhere to the endothelium in the initial stages of atherosclerosis or perhaps it is that the research to date has not been designed with this investigation in mind. By looking at published research into inhibition of platelet binding it may be possible to extrapolate further which of these is most likely to be correct.

Table 7: Evidence for Platelet Adhesion to Endothelial Cells in the Initial Stages of Atherosclerosis

Animal	Detail	Reference
Primates (human)	No evidence for platelet adhesion seen Mural thrombi in children	(Millonig et al., 2002, Emeson and Robertson, 1988, Stary, 1990, Spurlock and Chandler, 1987)
Primates (non-human)	Platelet adhesion in the initial stages of atherosclerosis No evidence for platelet adhesion	(Faggiotto et al., 1984a, Faggiotto and Ross, 1984b) (Stary and Malinow, 1982)
Rabbits	Adherent leukocytes for type 1 & type 2 lesion, but no adherent platelets Platelet adhesion to the endothelium in rabbit aorta Platelet adhesion prior to fatty streak formation	(Walker et al., 1986) (De Meyer et al., 1999) (Theilmeier et al., 2002)
In vitro (HUVECs on fibroblasts)	Platelet adhesion was the same for diabetic platelets and controls	(Nivelstein et al., 1991)
In vitro (BVEC)	Increased adhesion of diabetic platelets	(Manduteanu et al., 1992)
Mice	Platelet adhesion to the endothelium prior to leukocyte adhesion and lesion formation Platelets adhered to early lesions, but mainly in conjunction with monocytes.	(Massberg et al., 2002) (Huo et al., 2003)

(Key: HUVECs - human umbilical endothelial cells; BVEC - bovine vascular endothelial cells; ApoE^{-/-} - apolipoprotein E deficient)

3.2.6.3. Inhibition of Platelet Binding

If platelets are indeed involved in atherogenesis then blocking platelet adhesion should reduce lesion formation. Therefore, by looking at the presence of lesions, and their progression, it should be possible to determine whether anti-platelet drugs have an effect. There are a number of anti-platelet drugs that are available to treat patients with conditions such as cardiovascular disease. These include aspirin, cilostazol and clopidogrel.

One such drug, aspirin, is used for the secondary treatment of cardiovascular disease. It reduces platelet adhesion by blocking the production of platelet thromboxane A₂ and prostaglandin via inhibition of the cyclo-oxygenase pathway, and therefore inhibits platelet activation. It also inhibits prostacyclin synthesis by the endothelium therefore, it might increase thrombus formation. Animal investigations into the effect of aspirin on atherogenesis suggest that it

may or may not inhibit lesion formation, but it does reduce the severity of atherosclerosis (Guo et al., 2006, Pick et al., 1979).

Monkeys and rabbits fed on a high cholesterol diet and treated with aspirin have been reported to have lesions that were less widespread, had fewer layers, reduced total area of coverage and that vessel narrowing was only seen in the control animals. There was, however, no significant reduction in lesion formation (Pick et al., 1979, Guo et al., 2006). This would suggest that aspirin reduced the severity of atherosclerosis. It is possible that this is due to the antioxidant effect of high dose aspirin. (Therapeutic doses of aspirin have been reported to reduce the effect of oxidative stress on brain and endothelial cells (Podhaisky et al., 1997, Bode-Boger et al., 2005, Guerrero et al., 2004).) By acting as an antioxidant aspirin could reduce the availability of ox-LDL and thereby reduce foam cell formation. Indeed, aspirin has been reported to decrease platelet expression of ox-LDL (Marwali et al., 2007). It has also been suggested that aspirin reduces the inflammatory effect of ox-LDL on the endothelium (Zhao et al., 2008).

Other research has suggested that aspirin does reduce lesion formation as well as area of lesion coverage, in rabbits and humans (Cyrus et al., 2002, Kouraklis et al., 2004, Li et al., 2007, Mittal et al., 1990). Treatment of low-density lipoprotein receptor deficient (LDLR^{-/-}) mice with low doses of aspirin led to a decrease in mean size of the lesions and reduced presence of macrophages. There was, however, an increased level of collagen and smooth muscle cells – which could result in an increased stability in advanced lesions.

This research suggests that although aspirin may not stop atherogenesis, it may reduce monocyte recruitment and therefore foam cell formation. This might be partly due to its antioxidant effects partly reducing endothelial dysfunction and therefore cell adhesion molecule upregulation rather than platelet adhesion.

Although aspirin blocks the production of thromboxane it does not block intermediates in prostanoid production, F2-isoprostane and hydroxeicosatetraenoic acids (HETEs). S18886, a thromboxane receptor antagonist, however decreases synthesis of these. S18886 has been reported to reduce monocyte and macrophage recruitment to primary lesions and fatty streaks as well as to advanced lesions in rabbits fed on a high cholesterol diet (Worth et al., 2005).

Treatment of Apo E^{-/-} mice with S18886 decreased lesion size and reduced soluble levels of both ICAM-1 and thromboxane B₂, while, celecoxib, another cyclo-oxygenase inhibitor, was found to nearly completely inhibit the formation of atherosclerotic lesions (Cayatte et al., 2000, Jacob et al., 2008, Zuccollo et al., 2005). There was a decrease in lesion area of 81 % associated

with administration of this compound. Administration of celecoxib following lesion formation had no effect on further development and advancement.

Another drug, cilostazol, a cAMP phosphodiesterase inhibitor, reduced intimal-media thickness in Japanese patients with type 2 diabetes (Mitsuhashi et al., 2004). Intimal-media thickness is thought to be a marker of the early stages of atherosclerosis and therefore it is possible that, by inhibiting platelet aggregation and increasing vasodilation, cilostazol inhibits platelet adhesion.

A pronounced reduction in lesion formation was also reported in rabbits that were administered with a cocktail of anti-platelet drugs, including the platelet ADP receptor inhibitors clopidogrel and ticlopidine as well as aspirin (Li et al., 2007). Damage to the vasculature was induced at week one. The rabbits were then fed on an atherogenic diet before being slaughtered after seven weeks. At this point the reduced formation of lesions, compared to the controls, was most pronounced in the animals treated with clopidogrel, as opposed to those treated with ticlopidine or aspirin. This reduction was associated with decreased expression and serum levels of P-selectin, ICAM-1, VCAM-1 and MCP-1.

Herbert and team (Herbert et al., 1993) also found that clopidogrel and ticlopidine reduced platelet adhesion to the subendothelium in vivo and in vitro, but that aspirin had no effect. These findings are supported by other investigations (Jawien et al., 2007, Evangelista et al., 2005). Treatment of Apo E and LDLR deficient mice with ticlopidine resulted in a reduction in lesion area and reduced adhesion of CD68⁺ macrophages and CD3⁺ T lymphocytes (Jawien et al., 2007). The lymphocytes present, instead of being widely dispersed within the lesion, were accumulated in the peripheral areas of the plaque. Clopidogrel reduced platelet-leukocyte adhesion, platelet P-selectin expression, and platelet-derived reactive oxygen species production in mice (Evangelista et al., 2005). Experiments carried out, both ex vivo in mouse models, and in vitro, using human platelets, produced the same results. Both clopidogrel and ticlopidine block one of the ADP (P2Y₁₂) receptors and therefore reduce platelet aggregation by inhibiting activation of the cell adhesion molecule GPIIb/IIIa. There does not appear to be any investigations that investigate GPIIb/IIIa specific anti-platelet drugs (abciximab, eptifibatide, tirofiban). Findings from such studies could highlight whether platelet adhesion, via GPIIb/IIIa (ICAM-1 or $\alpha_v\beta_3$) is involved in lesion formation.

Other anti-platelet drugs have also been investigated. Reduced lesion formation was found in animal models treated with drugs that inhibit platelet degranulation and thrombus formation sulfinpyrazone and dipyridamole (Dembinska-Kiec et al., 1979, Grodzinska and Dembinska-Kiec, 1980, Landymore et al., 1988, Mittal et al., 1990). Dogs fed on a high cholesterol diet and treated with dipyridamole also exhibited a significant reduction in

intimal thickness. Combined treatment with aspirin also reduced intimal thickness, but no more so than dipyridamole on its own. The combined treatment did, however, reduce platelet count and thromboxane B₂ concentrations. Another study, however, found increased lesion formation following treatment with dipyridamole in rabbits (Dembinska-Kiec et al., 1979). The differences in results suggest that there are variations between animal models and therefore these findings be different again in humans. It would, however, be interesting to establish whether drugs that block platelet GPIIb/IIIa had a significant effect on lesion formation. These drugs would give a much clearer idea of whether platelets are involved. Therefore, by comparing these results with those from patients that lack specific platelet cell adhesion molecules or have abnormalities in platelet structure or function it may be possible to gain a better understanding.

Many of the anti-platelet drugs show a reduction in lesion formation. They do not, however, stop lesion formation altogether, even when a cocktail of these pharmaceuticals are used. This may well be because they do not stop upregulation of all the cell adhesion molecules that are associated with platelet-endothelial interactions and therefore platelet adhesion still takes place. The reduction seen may well be a consequence of the drugs having anti-inflammatory properties or unknown actions on the endothelium and leukocytes. If this is the case then, the reduction in lesion formation may not be the result of reducing platelet interactions. It is too early to tell whether new anti-platelet drugs, such as prasugrel and ticagrelor, which are likely to replace pre-existing treatments will result in a reduction in atherosclerosis. Therefore, this information currently available cannot, on its own, be used to determine whether my hypothesis is correct.

Table 8: Evidence for Reduced Lesion Formation as a Result of Anti-Platelet Therapies

Treatment	Detail	Reference
Aspirin	Coronary atherosclerosis less widespread and lesions were less obstructive in monkeys Low dosage decreased lesions and macrophage presence Decreased layers of foam cells, lesion distribution, coverage, and formation in rabbits & humans No effect on lesion formation	(Pick et al., 1979, Li et al., 2007, Cyrus et al., 2002, Kouraklis et al., 2004, Guo et al., 2006, Mittal et al., 1990, Napoli et al., 2002, Paul et al., 2000, Tous et al., 2004) (Schulz et al., 2008)
Ticlopidine	Decreased platelet adhesion to damaged vessels Decreased lesion area, CD68 and CD3 adhesion	(Herbert et al., 1993) (Jawien et al., 2007)
Clopidogrel	Decreased platelet adhesion and lesion formation in rabbits Decreased platelet-leukocyte adhesion, P-selectin, and platelet-dependent ROS production	(Herbert et al., 1993) (Evangelista et al., 2005)
Celecoxib	Decreased lesion formation in Apo E ^{-/-} mice	(Jacob et al., 2008)
Cilostazol	Reduced IMT	(Mitsuhashi et al., 2004)
S18886	Smaller lesions, decreased soluble ICAM-1 and TxB ₂ Decreased monocyte recruitment to primary lesions and fatty streaks in rabbits	(Cayatte et al., 2000) (Worth et al., 2005)
Dipyridamole	Decreased lesion and intimal thickness formation Increased lesion formation in rabbits and humans Combined treatment with aspirin decreased platelet count and TxB ₂ levels	(Harker et al., 1976b) (Dembinska-Kiec et al., 1979, Mittal et al., 1990) (Landymore et al., 1988)
Sulfinpyrazone	Decreased lesion formation	(Grodzinska and Dembinska-Kiec, 1980, Mittal et al., 1990)
prasugrel, ticagrelor, cangrelor and ProCorde GmbH 15 (PR-15)	No evidence found	None

(Key: ROS - reactive oxygen species; Apo E^{-/-} - apolipoprotein E deficient; IMT - intimal-medial thickness; ICAM - intercellular adhesion molecule; TxB₂ - thromboxane B₂)

3.2.6.4. Platelet Abnormalities

Another way of investigating the role of platelet adhesion in atherosclerosis is by looking at patients who have platelet defects that might result in abnormalities in platelet adhesion. There are a number of genetic and acquired conditions that affect platelets including genetic disorders that are associated with specific cell adhesion molecules or receptors, and acquired disorders associated with drugs. A literature review of these platelet abnormalities and the association with atherosclerosis was carried out.

Von Willebrand disease is a heterogeneous disorder that has a large number of variations, all of which are associated by genetic inheritance of changes in structure, function, or concentration of the cell adhesion molecule von Willebrand factor. This disease has been divided into three types (with associated variants). Type 1 is associated with decreased levels of VWF. This is the mildest and most common form of this disease. Type 2 is divided into groups dependent on which defects are present. It incorporates changes in multimer lengths and affinities for GPIb and factor VIII resulting in increased platelet adhesion. Type 3, on the other hand, is associated with almost complete deficiency of VWF.

Atherosclerosis has, however, been found in patients with von Willebrand disease (Silwer et al., 1966, Sramek et al., 2004). In type 3, the most severe form, atherosclerosis was found, even though the patients were almost completely deficient in VWF (Sramek et al., 2004). Intimal thickness in type 2B patients was reported to be the same as in atherosclerotic matched controls (Bilora et al., 2007). Lesions were, however, not reported. As type 2B is associated with increased affinity of VWF for GPIb/V/IX I would have expected that there would be an increase in atherosclerosis. Both studies only included information from a relatively small cohort, less than 50 VWD patients, but it would appear that the lack of VWF-GPIb adhesion does not deter lesion formation and an increase does not show accelerated lesion formation.

Further investigations have been done using animal models of von Willebrand disease, most notably in pigs (Fuster et al., 1982, Fuster et al., 1978, Griggs et al., 1981, Methia et al., 2001). Fuster and colleagues have been working with von Willebrand pigs for a number of years (Badimon et al., 1985, Badimon et al., 1989bb, Fuster et al., 1978, Bowie and Fuster, 1980, Badimon et al., 1989aa, Fuster et al., 1985, Fuster et al., 1977, Fuster and Bowie, 1978, Fuster et al., 1982). They initially detailed research that indicated that pigs were less inclined to develop atherosclerosis than controls in 1977. Since that point they have carried out a number of trials that have investigated the role of their diet in atherogenesis, the composition of fatty deposits and platelet adhesion.

Their investigations into spontaneous lesion formation found that seven of the eleven control pigs developed atherosclerotic lesion by the time of their slaughter. Five of these exhibited a maximum lesion diameter of greater than 2mm. In type 3 von Willebrand disease animals, three out of eleven had lesions, only one had a lesion with a diameter of greater than 2mm. It was reported, however, that there were flat fatty lesion in seven of the eleven of these pigs, but none in the controls. These flat lesions are not found in humans therefore this could suggest that the process of lesion formation or initiation is different in von Willebrand disease pigs. In high-fat, high cholesterol experiments, however, these flat fatty lesions were also seen in four of the controls. All of these animals had raised fatty and fibrous plaques. In contrast only three of the seven von Willebrand diseased pigs presented raised fatty plaque, with one of these also having raised fibrous plaque. All but one also exhibited flat fatty lesion. This would suggest that inhibition of VWF-dependent platelet adhesion does not stop foam cell formation but that it may slow the progression into raised lesions, and therefore more advanced atherosclerosis.

A later investigation found that the mean lesion coverage was less in type 3 von Willebrand disease pigs than the controls (Fuster et al., 1982). In controls 28% of the vasculature exhibited lesions. In von Willebrand disease pigs there was a significant reduction to only seven percent coverage. Both groups were fed the same non-fatty diet for 4 years. Potential genetic involvements were investigated by carrying out transplantations of aortic segments. Normal segments were transposed. The resulting atherosclerosis amounted to 20% coverage. Transplantation of a normal segment into a von Willebrand disease pig, on the other hand, resulted in only 4% coverage – a notable decrease. This would suggest that there are no differences in vessel genetics that allow for reduced lesion formation.

Investigations were also carried out to look at heterozygous and homozygous von Willebrand disease pigs (Griggs et al., 1981, Badimon et al., 1985). Griggs and colleagues reported that there was no significant decrease in tissue lipid deposits (as determined by Sudan dye staining) for either of the disease types. The homozygous animals did, however, exhibit a significant decrease in fibrous plaque formation. This, on the whole supported the finding of Badimon et al., although they did report that the heterozygous VWD pigs had a similar percentage of raised fatty and fibrous plaque as control pigs. Both heterozygous and controls had a significantly higher percentage of lesion coverage than homozygous von Willebrand disease pigs.

There have been criticisms of these investigations. One of these is that Apolipoprotein B100 polymorphisms can be found in swine. Changes to these genes have been linked with higher blood cholesterol (Griggs et al., 1981,

Nichols et al., 1992). Feeding these animals on a high-fat, high-cholesterol diet would increase foam cell formation and therefore make the investigations using the hypercholesterolaemia diet unreliable. The reports, however, looking at spontaneous lesion formation do not fall into this category.

Mice have also been used as a model of type 3 von Willebrand disease (Methia et al., 2001). VWF^{-/-} and low-density lipoprotein receptor LDLR^{-/-} mice were crossed to produce a mouse model that was susceptible to atherosclerosis. After 8 weeks of an atherogenic diet, the mice were found to have 40% less mean plaque area. This decrease in lesion formation was still apparent at 22 weeks, but had normalized by 37 weeks, at the point in which fibrous lesions occur in the control animals. Therefore, it can be proposed that VWF is required for lesion initiation but is not involved in progression to fibrous plaque formation. This is supported by the observation that macrophage infiltration was lower in LDLR and VWF deficient mice than in the control animals. It is possible that this is a result of reduced P-selectin associated with this mouse model (Denis et al., 2001). The extent of reduction, nevertheless, does not appear to correlate with the reduction in P-selectin alone.

Glanzmann thrombasthenia is associated with genetic defects in platelet GPIIb/IIIa and $\alpha_v\beta_3$ receptors. A number of mutations have already been determined that result in changes to transportation of the GPIIb/IIIa complex to the surface, surface expression, ligand binding, and increased sensitivity to calcium. In one study, seven Iraqi-Jewish patients with Glanzmann thrombasthenia who were 46 years of age or over underwent screening for atherosclerosis. Six of these seven patients had signs of the early stages of atherosclerosis. This was determined by the presence of plaque and intermedial thickness. A pathologist would normally expect to find more advanced stages of atherosclerosis in patients of this age. Five of these patients had no GPIIb/IIIa or $\alpha_v\beta_3$ adhesion molecules (Shpilberg et al., 2002). It is therefore possible that reduced lesion formation was a direct result of a reduction in the amount of platelet GPIIb/IIIa and $\alpha_v\beta_3$ associated binding to the endothelium.

Abnormalities in other platelet cell adhesion molecules have also been investigated, including β_3 and α_2 integrins. Weng and colleagues also looked at the role of β_3 integrins in lesion formation (Weng et al., 2003). They developed β_3 , apolipoprotein E ($\beta_3^{-/-}$ apo E^{-/-}), and β_3 , low-density lipoprotein receptor ($\beta_3^{-/-}$ LDLR^{-/-}) deficient mice. Both mouse models were fed on an atherogenic diet, with those mice without β_3 receptors having greater lesion development than their litter mates. While there was no significant changes were seen in lesion formation in $\alpha_2^{-/-}$ Apo E^{-/-} when compared with wild type and $\alpha_2^{+/+}$ Apo E^{-/-} mice. Macrophage infiltration also remained the same (Grenache et al., 2003).

Fibrinogen deficiency has also been reported to lead to substantial decreases in lesion formation in apolipoprotein(a) (Apo(a)) active and fibrinogen deficient mice (Apo(a)Fib^{-/-}) (Lou et al., 1998). This mouse model allows for in vivo investigations into the contribution of Apo(a) and fibrinogen in lesion formation. A large increase was indicated both by fatty deposit staining and by measurement of the total lesion area. Another study has, however, reported that the lack of fibrinogen increased the formation of lesions (Iwaki et al., 2006). The investigation used low density lipoprotein (LDLR) / apolipoprotein B-100 complex (APOBEC1) / fibrinogen deficient mice, to look at the effect of fibrinogen on cholesterol driven lesion formation. The rate of formation was not increased, just the surface area and size of the plaque, suggesting that atherogenesis is initiated faster than in the controls.

Atherosclerosis has been reported in a number of conditions associated with platelet abnormalities. These abnormalities relate to a specific function of platelets and therefore do not completely rule out platelet adhesion to the endothelium in these patients. Indeed, you would expect some platelet interactions, and therefore a significant reduction in atherogenesis if platelet adhesion was involved. The published information does, on the whole, suggest that lesion formation is reduced in a number of these conditions. It is difficult, however, to determine the severity of the abnormalities, in relation to platelet adhesion. The evidence to date, as a whole, leaves a lot of unanswered questions. It would suggest that platelets may have a role in initiating lesion formation but that it is possible that they are not an absolute requirement for atherogenesis. It is not clear, however, whether platelet adhesion occurs prior to lesion formation under diabetic conditions. I believe that it is clear to see that further investigation is required to establish whether this is the case.

Table 9: Details of Platelet Pathologies and Evidence for Decreased Lesion Formation

Abnormality	CAM	Detail	Reference
VWD in humans	VWF	Atherosclerosis found in all patients with VWD. The incidence of atherosclerosis is the same as controls	(Silver et al., 1966, Sramek et al., 2004, Bilora et al., 2007)
VWD pigs	VWF (& VIII)	Decreased lesion formation VWF was not involved in lesion formation in pig aortas Heterozygous VWD pigs did not have reduced lesions	(Fuster et al., 1978, Fuster et al., 1982) (Griggs et al., 1981) (Badimon et al., 1985)
VWD mice	VWF	Reduced lesion formation. Decreased monocyte infiltration	(Methia et al., 2001)
VWD or deficiency in VIII or IX	VWF, factor IX	No effect on lesion formation or coagulation (haemophilia, moderate & severe haemophilia, mild haemophilia & VWD, mild VWD)	(Sramek et al., 2001)
Glanzmann Thrombasthenia	GPIIb, GPIIIa & $\alpha_v\beta_3$	Atherosclerosis was present	(Shpilberg et al., 2002)
β_3 deficiencies	GPIIb/IIIa & $\alpha_v\beta_3$	Increased lesion formation	(Weng et al., 2003)
Bernard-Soulier	GPIb	Patient had atherosclerosis – could not find any other instances of lesions in GPIb deficiencies	(Humphries et al., 1992)
α_2 deficiencies	GPIIb/IIa	No significant difference	(Grenache et al., 2003)
Abnormal fibrinogen	Fibrinogen	Decreased lesion formation seen in Apo (a)/Fib ^{-/-} mice Increased lesion formation in mice	(Lou et al., 1998) (Iwaki et al., 2006)

(Key: CAM – cell adhesion molecule; VWD - von Willebrand disease; T3 - type 3; Apo (a)/Fib^{-/-} - apolipoprotein A/fibrinogen deficient)

3.3.What can be concluded?

Although it was initially thought that the endothelium was passive, with platelet adhesion only resulting from endothelial denudation, it is now clear that this is not the case. Platelet adhesion to the vessel walls has been established as involving interactions with the sub-endothelium, the extracellular matrix and with the endothelium. Adhesion is known to occur when either, or both, platelets or the endothelial cells are activated, as a result of upregulation of cell adhesion molecules.

There has been a lot of research into platelet and endothelial cell adhesion molecules, as well as their associated ligands, over the last couple of decades. There is also a large amount of literature covering cell adhesion molecule and receptor interactions. These have suggested that adhesion molecule interactions differ based on whether platelets and / or endothelial cells are activated. There are also some discrepancies, which are probably a result of different methodologies being used, (including cell preparation or culture), as well as what cells or animal models used. It is still clear, however, that endothelial $\alpha_v\beta_3$, GPIb, and platelet GPIIb/IIIa are key receptors, with fibrinogen, and VWF acting as important ligands.

Platelet rolling has been associated with GPIb, PSGL-1, and P-selectin. Firm adhesion, on the other hand, has been reported to require GPIIb/IIIa, ICAM-1, and $\alpha_v\beta_3$. It is thought that platelet adhesion is initiated by tethering and rolling, followed by firm adhesion. Whether this research mimics physiological interactions in humans is unclear. Indeed, it is unlikely that any one specific receptor-counter-receptor interaction holds the key, rather the contribution of many. Therefore, for platelet adhesion to be involved in the initiation of atherosclerosis, an array of platelet and endothelial cell adhesion molecules may be involved. As such, I proposed to stay away from looking at specific cell adhesion molecules for my studies into platelet adhesion in the initial stages of atherosclerosis.

A number of papers have looked at the development of lesions. The majority of these have not reported platelet adhesion. The lack of information may suggest that they have no role. It could, however, just be the result of studies concentrating on leukocyte, particularly monocyte, interactions because they are a pre-requisite for lesion formation. Otherwise, a lot of research has concentrated on lesion development without any reference to how they develop. Therefore, a lot of studies will not have looked for the presence of platelets.

There are, however, a few reports of platelets adhering to the endothelium prior to lesion formation in animal studies and also at sights known to be key areas for lesion development in humans. Obviously, these may well be a result of the procedures used to collate this information. If, however, platelets were indeed involved it would be a fair assumption to make that blocking platelet adhesion or abnormalities that result in decreased activation or adhesion would reduce lesion formation.

The evidence in the literature is not very expansive. From what information there is, it is possible to conclude that there may well be a reduction in lesion formation but that either lesion formation still occurs. The studies looking at patients who regularly take anti-platelet drugs are likely to have developed lesions, based on the likelihood that they were placed on these drugs to treat cardiovascular disease or other conditions associated with atherosclerosis. Investigations into patients with platelet abnormalities, on the other hand, may well not have complete inhibition of platelet activation and / or adhesion. From this, I believe that it is clear to see that there is a lack of information already available that can determine whether platelet adhesion is an absolute requirement for lesion formation in diabetic conditions.

My proposed research aimed to bridge this gap. The aim was to compare, under similar laboratory conditions, various methods of investigating platelet adhesion to the endothelium. As such, it was hoped that a clearer understanding, of whether platelets adhere to the endothelium in diabetic conditions, could be achieved.

The research was to centre on cultured endothelial cells (HUVECs), although it was hoped to acquire diabetic tissue for comparison. Platelet adhesion was to be determined by first incubating the cells with high glucose and / or various AGEs and then using flow cytometry or immunocytochemistry to visualise any platelet-endothelial interactions.

Dynamic flow studies were to be carried out to complement these studies. It was hoped that by using this technique it would allow for investigation into whether the processes involved for staining and fixing had any effect as well as to determine what difference flow conditions had on platelet interactions.

I would have liked to be able to carry out an investigation into platelet adhesion in patients who suffered premature death. (One similar to that detailed by Katsuda and colleagues but with collection and imaging that would allow for any platelet adhesion to be determined (Katsuda et al., 1992).) This would have allowed me to see whether platelet adhesion was seen prior to and during lesion formation. The university facilities meant that this was not possible but, I believe that this type of research would be required to give a more accurate view.

To expand on this research, I also proposed to look at platelet-leukocyte interactions with the endothelium. It is clear that leukocytes, in particular monocytes, are required for foam cell formation, and therefore lesion development. Platelets have cell adhesion molecules for leukocyte so it is likely that they interact. It may well be that platelet adhesion to leukocytes is an initiating factor for the creation of foam cells. Therefore, adhesion studies, using platelets and leukocytes were proposed.

4. Platelet-Leukocyte Complexes Increase Lesion Formation in the Initial Stages of Atherosclerosis

4.1. Introduction

Platelet adhesion to the endothelium cannot alone account for atherosclerosis. For lesions to develop, foam cell formation is required. Therefore, monocyte adhesion and transmigration into the subendothelium is required. Leukocytes have cell adhesion molecules for both platelets and endothelial cells. (From the previous chapter and from Table 10, it was clear to see that both platelets and endothelial cells express cell adhesion molecules that act as receptors for leukocytes; for instance Mac-1 and LFA-1.) It is possible that leukocyte interaction with platelets accelerates lesion formation by amplifying transmigration and differentiation into macrophages. Indeed, evidence that platelet-leukocyte interactions may hold the key has already been mentioned in the previous chapter. It was reported that platelets rarely adhered in isolation and that platelets adhered to monocytes. These monocytes were reported to interact with the endothelium (Huo et al., 2003). I therefore hypothesize that platelet leukocyte complexes increase monocyte transmigration, above that normally expected, and as such contribute to accelerated atherosclerosis in diabetics. As such, I proposed to investigate whether platelet adhesion to leukocytes increases their adhesion to the endothelium under diabetic conditions.

Table 10: Leukocyte Cell Adhesion Molecules

Name	Receptor for	Leukocyte	Reference
LFA-1	ICAM-1 ICAM-2 ICAM-3 JAM-A	Lymphocytes, monocytes, macrophages, and granulocytes	(Bell et al., 1998, Casasnovas et al., 1999, Jun et al., 2001, Ostermann et al., 2002)
Mac-1	Fibrinogen GPIIb/IIIa GPIb α JAM-C ICAM-1 iC3b Factor X NIF HMW kininogen	Neutrophils, monocytes, eosinophils, baophil and NK cells	(Beller et al., 1982, Chavakis et al., 2003, Santoso et al., 2002, Wang et al., 2005, Altieri and Edgington, 1988, Kawabata et al., 2002, Diamond et al., 1990, Rieu et al., 1994, Wright et al., 1988)
P150.95	JAM-C Heparin iC3b Fibrinogen Denatured proteins	High levels on monocytes and macrophages but also found on other leukocytes	(Loike et al., 1991, Santoso et al., 2002, Vorup-Jensen et al., 2007, Micklem and Sim, 1985, Davis, 1992)
VLA-4	VCAM-1 E-selectin, Fibronectin Thrombospondin	Lymphocytes	(Elices et al., 1990) Shimizu <i>et al.</i> 1991 Wayner <i>et al.</i> 1989 Yabkowitz <i>et al.</i> 1993
VLA-5	Thrombospondin	Lymphocytes and monocytes	Yabkowitz <i>et al.</i> 1993
L-selectin (CD62L)	Sialyl Lewis(x) PSGL-1 Carbohydrate ligands P- & E-selectin	Leukocytes	(da Costa Martins et al., 2004, Finger et al., 1996, Foxall et al., 1992, Walcheck et al., 1996)
PECAM-1	$\alpha_v\beta_3$	Leukocytes	(Ashman and Aylett, 1991)
PSGL-1	P-selectin E-selectin	Neutrophils, monocytes, most T lymphocytes and some B lymphocytes	(Zou et al., 2005, Moore et al., 1995)
JAM-A	?	Leukocytes	(Williams et al., 1999)
JAM-C	JAM-A	Leukocytes	(Liang et al., 2002)

(Key: LFA-1 - lymphocyte function-associated antigen, $\alpha_L\beta_2$ or the CD11a/CD18 complex; ICAM - intracellular adhesion molecule; Mac-1, $\alpha_M\beta_2$ or the CD11b/CD18 complex; JAM - junctional adhesion molecule; iC3b - inactivated complement compound 3b; NIF - hookworm neutrophil inhibitory factor; p150.95, $\alpha_X\beta_2$ or the CD11c/CD18 complex; VLA - very-late antigen; VLA-4, $\alpha_{IV}\beta_1$ or the CD49d/CD29 complex; VCAM - vascular cell adhesion molecule; VLA-5, $\alpha_v\beta_1$ or the CD49e/CD29 complex; PSGL - P-selectin glycoprotein; PECAM - platelet endothelial cell adhesion molecule)

4.2. My Proposed Research

To determine whether platelet-leukocyte interactions with the endothelium were required for lesions to develop in diabetic conditions I proposed to expand on adhesion studies. As in Chapter 3, cultured endothelial cells were to be incubated with high concentrations of glucose and / or AGEs prior to exposure to a suspension of platelets and leukocytes. Platelet-leukocyte interactions with the endothelium were then to be investigated by flow cytometry and immunocytochemistry, using the techniques already optimized for platelet adhesion studies. Dynamic flow studies would also allow for real time interactions as well as to determine whether platelets increased leukocyte adhesion.

4.2.1. Flow Cytometry

Flow cytometry detection of platelet and leukocyte interactions with cultured endothelial cells was to be achieved by using the method detailed in Chapter 3, with the inclusion of leukocytes. To achieve this, erythrocytes were to be lysed from whole blood samples. Platelets (both ADP-activated and non-activated) and endothelial cells were then to be stained, as previously described, with calcein-AM or propidium iodide. Leukocytes would then be labelled with a tandem conjugated anti CD45 antibody (Peridinin chlorophyll protein conjugated with the Cy5.5 dye [PerCP-Cy] allowing for reduced compensation, less leaching, and less background for monocytes/macrophages than when using PerCP or PE-Cy.). This would allow for the detection of FL-1 scatter to represent platelets, FL-2 filter scatter for endothelial cells, and FL-4 filter scatter for leukocytes. As such, this would allow for determining whether endothelial incubation with high glucose and/or AGEs increased leukocyte-endothelial, platelet-endothelial, or platelet-leukocyte interaction with the endothelial cells in comparison with the controls.

Both diabetic and non-diabetic blood were to be used for these adhesion studies. This was so that it could be ascertained whether changes to platelets associated with long-term diabetes (such as hyperglycaemia and AGEs) could affect leukocyte adhesion. These changes were not expected to be seen from 30 minute incubation in conditions of high glucose and AGEs and therefore needed exploring further.

4.2.2. Immunocytochemistry

To compare the flow studies with adhesion to a monolayer, immunocytochemistry was planned. The protocol determined for platelet adhesion studies was to be expanded to allow for detection of adhered leukocytes. The platelet and leukocyte were to be collected as for the flow cytometry studies and then administered to wells containing near confluent cultured endothelial cells on coverslips. (Again both diabetic and non-diabetic platelets were to be administered.) Following fixing, platelets would be labelled with FITC-CD42b

and leukocytes with Texas red-CD45. The endothelial cell nuclei would be stained with DAPI and a mounting medium applied. This would allow for visualization of whether platelets and leukocytes adhered to the monolayers under diabetic conditions.

4.2.3. Dynamic Flow

Adhesion was also to be investigated in real-time. With the use of a flow chamber, video analysis would allow for determination of whether blood flow affected interactions with the monolayer or tissue sample. Again, by adapting the procedure, detailed in Chapter 3, to pass both platelets and leukocytes across the endothelium, at various flow rates, it would be possible to see whether diabetic conditions resulted in increased leukocyte adhesion as a result of platelet interaction. A comparison could be made between leukocyte adhesion with or without the presence of platelets by lysing the red blood cells and counting the leukocytes present at various time intervals. Quantifying the amount present is much easier with this method than using flow cytometry and immunocytochemistry. These rely on quantifying by intensity of the fluorescence. With the flow cytometer, it was possible to gate a specific population and find out how long it takes to reach a certain cell count. This, however, is not that accurate because the population may contain cellular debris as well as some cells from other populations.

4.3. Research so Far

There has been some research published that has investigated the role of platelets in leukocyte adhesion to the endothelium. This research is briefly reviewed here and includes cell culture as well as animal studies to investigate leukocytes, platelets and endothelial complexes. Studies have also looked into cell adhesion molecules involved in these interactions.

The combined interaction of platelets, leukocytes and the endothelium has been reported by several research groups (Hirafuji and Shinoda, 1991, Huo et al., 2003, Ishikawa et al., 2007, Ishikawa et al., 2004, Ludwig et al., 2004, Massberg et al., 2003, Mine et al., 2001). In a mouse model of atherosclerosis, it was found that platelet adhesion to the endothelium occurred via monocytes (Huo et al., 2003). In this study, the carotid bifurcation and external branch were exposed prior to perfusion of calcein labelled platelets and injecting rhodamine 6G to label leukocytes. Leukocyte-platelet interactions with the vessel wall were observed by intravital microscopy. Indeed, platelets rarely interacted with the endothelium individually. When isolated monocytes were administered, there was an increase in their arrest in the presence of activated platelets. This is also supported by other studies where the presence of platelet-mononuclear leukocyte conjugates has been reported to increase platelet recruitment and increased leukocyte arrest on the endothelium in the presence

of activated platelets (Massberg et al., 2003, Mine et al., 2001, da Costa Martins et al., 2004, Hirafuji and Shinoda, 1991, Huo et al., 2003, Ishikawa et al., 2004).

This does not necessarily intimate that platelet-leukocyte-endothelium (PLECs) or leukocyte-platelet-endothelial complexes (LPECs) occur, but that there is some sort of interaction, or cross talk, between them in some way that result in increased monocyte adhesion to the endothelium. There is, however, evidence to support the theory of PLECs and LPECs (Schulz et al., 2007, Ishikawa et al., 2007, Ishikawa et al., 2004, Diacovo et al., 1996, Bernardo et al., 2005).

PLECs were clearly shown by intravital fluorescence microscopy (Ishikawa et al., 2007, Ishikawa et al., 2004). Following bilateral common carotid artery occlusion in mice, a few LPECs were found but PLECs were much more numerous. There does not, however, appear to be evidence from other sources to support these findings.

Leukocytes have been reported to adhere to endothelial-bound platelets by a number of different research groups (Bernardo et al., 2005, Diacovo et al., 1996, Schulz et al., 2007). Schulz and colleagues investigated interactions in mice as well as with cultured cells (Schulz et al., 2007). Similar experimental conditions were used as those of Ishikawa et al. (Ishikawa et al., 2004, Ishikawa et al., 2007). However, different types of mice were used and different techniques were chosen for labelling leukocytes. This study concentrated on monocyte specific adhesion and therefore it is possible that monocytes adhere to the endothelium via platelets while other types of leukocytes interact directly.

Cell culture studies have also shown that LPECs occur (Schulz et al., 2007). Flow cytometry was used to show adhesion to IFN- γ (interferon gamma) and TNF- α (tumour necrosis factor alpha) activated endothelial cells (Schulz et al., 2007). This was supported by dynamic flow studies using thrombin activated platelets and non-activated neutrophils, as well as histamine stimulated endothelial cells and leukocytes (Diacovo et al., 1996). All these in vitro studies used HUVECs

Interestingly, Schulz and colleagues (Schulz et al., 2007) stated that leukocyte adhesion to HUVECs was observed mainly at sites of platelet adhesion and inhibition, using platelet blocking antibodies or the absence of platelets, significantly reduced leukocyte adhesion under arterial flow conditions (1000/s). With venous flow conditions (100/s), however, leukocyte adhesion was not dependent on platelet adhesion.

A number of adhesion molecules have been reported to be involved in PLEC or LPEC interactions, including the selectins, PSGL-1, ICAM-1, Mac-1, LFA-1, VLA-4, and VWF (da Costa Martins et al., 2007, da Costa Martins et al., 2004, Alvarez et al., 2004, Barry et al., 1998, Bernardo et al., 2005, Diacovo et al., 1996, Kirton and Nash, 2000, Schulz et al., 2007).

Leukocytes have also been observed adhered to HUVEC bound platelets under high shear flow conditions (Bernardo et al., 2005, Diacovo et al., 1996). Diacovo and team found that neutrophil adhesion was Mac-1 dependent and that ICAM-2 and LFA-1 were not involved. Adhesion was reported to decrease with increasing flow rates (from 0.75 to 35 dyn/cm²). On the other hand, Bernardo and colleagues looked at higher shear rates (20 and 40 dyn/cm²). Under these conditions, leukocytes interacted and rolled on platelets adhered to mouse mesenteric venule endothelium-bound ultra large von Willebrand factor (ULVWF) via P-Selectin.

P-selectin has been extensively reported to be involved in platelet-leukocyte interactions (da Costa Martins et al., 2007, Alvarez et al., 2004, Diacovo et al., 1996, Kirton and Nash, 2000, Schulz et al., 2007, da Costa Martins et al., 2004). Other cell adhesion molecules have, however, limited published information to determine their involvement.

Cell culture studies have shown that monocyte adhesion to HUVECs could be inhibited via platelet P-Selectin and PSGL-1 (da Costa Martins et al., 2007, da Costa Martins et al., 2004, Schulz et al., 2007). A reduction in adherence was also found by blocking E-Selectin, L-Selectin and Mac-1, but not LFA-1 or ICAM-2 (da Costa Martins et al., 2007, da Costa Martins et al., 2004, Diacovo et al., 1996). In human microvascular endothelial cells, however, it has been reported that blocking Mac-1 and LFA-1 reduces neutrophil interactions with endothelium-bound platelets (Kirton and Nash, 2000).

One other study, interestingly, highlighted the variation in cell adhesion molecule contribution associated with vessel type (Alvarez et al., 2004). They found that mice infused with anti-P-selectin and anti-E-selectin antibodies exhibited a decrease, to basal level, in platelet-leukocyte adhesion in arterioles, suggesting the role of the endothelium in platelet interactions with leukocytes. There was no effect reported in venules.

This poses the question as to whether the type of interaction seen is dependent on the cells used and the experimental design. For instance, the type of cells used, whether the investigations are in vivo or in vitro, the antibodies used, and the detection methods. However, my hypotheses were not dependent on determining this, instead whether platelet adhesion could allow for increased leukocyte adhesion.

Other research has investigated whether platelet depletion or thrombocytopenia affects leukocyte adhesion (Nishijima et al., 2004, Merhi et al., 1997, Kovacs and Caen, 1979). Anesthetised pigs and rats given an anti-platelet serum showed a reduction in leukocyte adhesion. Other studies, however, suggest that thrombocytopenia resulted in increased leukocyte adhesion (Kovacs and Caen, 1979). The experimental design, however, was quite different. Firstly one used pigs and the

other rats for the research. They used different sedation and anesthetic drugs. One looked at radio labelled neutrophils while the other just counted granulocyte adhesion using a microscope and they used different time frames for anti-platelet serum administration prior to analysis. This makes comparison very difficult and does not really help to determine whether the discrepancies are a result of the animal model used or the experimental procedure. They also do not determine whether monocyte adhesion is increased, which is of most interest for my research.

There also does not appear to be any studies that have concentrated on investigating increased leukocyte adhesion to the endothelial as a result of platelet adhesion under diabetic conditions. My research would therefore allow for determination as to whether increased platelet-leukocyte interactions with the endothelium occur following endothelial exposure to diabetic conditions – high glucose and / or AGEs. If the research points to this then it is likely that platelets have a key role in the initiation of accelerated atherosclerosis. It may also highlight whether physical interaction is required between platelets and leukocytes or whether there is some sort of cross talk that leads to increased leukocyte interaction. This will be ascertained by seeing whether the presence of platelets and leukocytes increase leukocyte adhesion compared with leukocytes on their own.

Table 11: Overview of Research Indicating Platelet-Leukocyte-Endothelial interactions

Adhesion Molecule	Detail	Reference
General	<p>Platelet adhesion to ECs when PMN are present</p> <p>Activated platelets caused leukocyte adhesion to ECs</p> <p>Platelet-leukocyte interactions increased leukocyte rolling on the endothelium in mice</p> <p>Platelets interacted with leukocytes that were arrested on the endothelium.</p> <p>Platelet-leukocytes rolled on endothelium <i>in vivo</i></p>	<p>(Hirafuji and Shinoda, 1991)</p> <p>(Ishikawa et al., 2004)</p> <p>(Huo et al., 2003)</p> <p>(Massberg et al., 2003, Mine et al., 2001, Ludwig et al., 2004)</p> <p>(Ishikawa et al., 2007)</p>
P-selectin	Platelets adhered to ECs and initiate the expression of platelet P-selectin. This results in adhesion of leukocytes	(Schulz et al., 2007)
L-selectin	PMCs adhered to the endothelium. This is reduced by mAb anti-L-selectin	(da Costa Martins et al., 2004)
P-selectin, PSGL-1, β_1 , β_2	Monocytes in PMCs have greater affinity for the endothelium	(da Costa Martins et al., 2007)
P-selectin, β_2 , α_4 , ICAM-1, E-selectin	P-selectin & β_2 are involved in arterioles, ICAM-2, β_2 & α_4 are involved in veins, none are involved in venules	(Alvarez et al., 2004)
P-selectin & Mac-1	P-selectin-Mac-1 interaction for platelet-leukocyte adhesion when the platelets were attached to the endothelium	(Diacovo et al., 1996)
P-selectin, Mac-1, LFA-1	Blocking P-selectin stopped leukocyte adhesion to bound platelets. Initial interaction was mainly by Mac-1. LFA-1 involved.	(Kirton and Nash, 2000)
P-selectin & VWF	Leukocytes adhered to bound platelets (ULVWF strings) under shear stress	(Bernardo et al., 2005)

(Key: ECs - endothelial cells; PMN - Polymorphonuclear; ICAM - intercellular adhesion molecule; LFA - lymphocyte function associated antigen; PMCs - platelet-monocyte complexes; PSGL - P-selectin glycoprotein ligand; VWF - von Willebrand factor; UL-VWF - ultra large von Willebrand factor)

4.4. What can be concluded?

Leukocytes, specifically monocytes, are recruited in the initial stages of atherosclerosis. Indeed, lipid phagocytosis by macrophages is a prerequisite for lesion formation. Leukocyte adhesion has been shown to occur when platelets are adhered to the endothelium and therefore it is possible that platelets may have a role to play in leukocyte recruitment. Platelet interaction with leukocytes under diabetic-like conditions was explored to ascertain whether they accounted for accelerated atherosclerosis in these circumstances.

My studies aimed to investigate whether platelet adhesion increased leukocyte adhesion to the endothelium under diabetic-like conditions. I also hoped to determine whether this was via platelet-leukocyte-endothelial, leukocyte-platelet-endothelial, some sort of cross talk between platelets and leukocytes, or a mixture of all of these. If increased leukocyte adhesion was observed it is likely that platelets are involved in lesion formation.

Published work, to date, highlights that the role of platelets in leukocyte adhesion in diabetic-like conditions has not been explored. It does, however, show that activated platelets, or platelets under high shear flow conditions, increase leukocyte affinity for the endothelium. Both platelet-leukocyte-endothelial and leukocyte-platelet-endothelial interactions have been reported. The precise nature of these interactions has not been resolved conclusively; however, blocking studies suggested that ICAM-1, E-Selectin, P-Selectin, PSGL-1, VWF, VLA-4, LFA-1, and Mac-1 may be involved.

From this research I hypothesized that diabetic-like conditions would lead to increased leukocyte adhesion in the presence of platelets. Whether this was as a result of direct interaction or as a result of cross-talk was to be determined and also, whether platelet activation under high shear rate conditions was required. To continue on from this research, I also proposed to determine what effect diabetic-like conditions would have on endothelial activation or dysfunction that would allow for increased lesion formation. This would allow a clearer understanding of what processes are involved in accelerated lesion formation associated with diabetics.

5. Diabetes Causes Accelerated Atherosclerosis by Increasing Endothelial Dysfunction

5.1. Introduction

Diabetes results in increased lesion formation. As such, this disease must increase leukocyte adhesion to the endothelium. It is believed that this is the result of endothelial dysfunction. There is no specific definition for the term endothelial dysfunction, but it is generally considered to be any changes that result in a pro-inflammatory, pro-adhesive, pro-thrombotic condition that is often associated with altered protein expression following stimulation with cytokines (and this is the basis for the definition used here) (Zimmerman *et al.* 1999). It is therefore a term used to encompass a number of disturbances in the normal function of the endothelium, including changes in vasomotor responses, permeability, proliferation, and cell adhesion molecule expression. There are a number of pathologies linked to endothelial dysfunction; these include hyperglycaemia, insulin resistance, advanced glycation end-product (AGE) formation, and oxidative stress (overviewed in Tables 12-14). Any one or a combination of conditions may be present at a specific point in time.

There are many reviews that discuss, in detail, endothelial dysfunction and its various causes (Caballero, 2003, Cai and Harrison, 2000, De Vriese et al., 2000, Esper et al., 2006). Research investigating the effects of hyperglycaemia and AGEs, due to diabetes, on various markers of endothelial dysfunction is concentrated on here, due to the link between diabetes and accelerated atherosclerosis.

Diabetes, both type 1 (T1DM) and type 2 (T2DM), is considered to be one of the main causes of endothelial dysfunction and reactive oxygen species (ROS) formation. It has been suggested that endothelial dysfunction in T2DM is likely to be caused by poor blood glucose control and increases in glucose metabolism via the polyol pathway. In T1DM endothelial dysfunction has also been linked with oxidative stress but what causes this is unknown. The exact cause of endothelial dysfunction in diabetes is still, however, in question.

In T2DM, exposure to higher than normal levels of blood glucose, over a prolonged time period, results in a condition known as glucose toxicity. Glucose toxicity is known to cause severe deterioration in the function and number of pancreatic β -cells (Robertson et al., 1992). It is therefore likely that high concentration of glucose will have an adverse effect on other tissues, including the endothelium.

Research suggests that hyperglycaemia does indeed affect the endothelium. High levels of glucose cause changes in vasomotor control, increased production of the reactive oxygen species, formation of glycation end products (AGEs), production of metalloproteinases (MMPs), and potentially irreversible increased endothelial permeability as well as monocyte adhesion (De Vriese et al., 2000, Donnini et al., 2003, Graier et al., 1999, Heitzer et al., 2001, Uemura et al., 2001).

Hyperglycaemia also results in an increase in glucose metabolism via the polyol pathway. (Under normal physiological conditions, glucose metabolism occurs, almost exclusively, via glycolysis.) This increase in flux results in a reduction in (Na^+ / K^+)ATPase activity due to activation of the PKC pathway and an increase in the NADH:NAD ratio (Brownlee, 2001). As such, it also increases production of oxygen-derived free radicals. Free radical production in diabetics is therefore higher. It has, however, not only been reported to be caused by an increased flux in the polyol pathway, but also due to increased glycation, decreased NO availability (due to increased peroxynitrite formation), and decreased eNOS activity (uncoupled eNOS has been found in diabetic patients, which is thought to be due to decreased availability of NO) (Jay et al., 2006).

Enhanced levels of circulating free radicals in diabetics have been linked with cellular damage. This is exacerbated by a decrease in the production and effect of antioxidants (Jay et al., 2006). The result is oxidative stress. Evidence suggests that oxidative stress is linked to the occurrence of complications associated with diabetes, including microvascular disease, cardiac complications and atherosclerosis (Jay et al., 2006, Heitzer et al., 2001, Aydin et al., 2001, Delbosc et al., 2005). Oxidative stress is known to result in endothelial dysfunction and therefore will result in an array of changes to the endothelium, including making it pro-adhesive. (Markers for oxidative stress and endothelial dysfunction in diabetics can be seen in Figure 13.) It is possible that it would result in platelet adhesion as well as increased monocyte interactions. To investigate whether this is likely, under diabetic conditions in vitro, various experiments were planned. These included looking at whether incubation of endothelial cells with high concentrations of glucose and / or AGEs resulted in changes to the viability of the monolayer, increased expression of cell adhesion molecules, pro-inflammatory and adhesive cytokines, as well as various reactive oxygen species. I hypothesized that a combination of these diabetic conditions would result in changes to all these markers of endothelial dysfunction and therefore it was likely that increased monocyte (and possibly platelet) adhesion was a direct result.

Table 12: Endothelial Dysfunction

Physiological	Pathological
Impaired NO function	Decreased vasodilation and increased platelet adhesion
Impaired prostacyclin production	Decreased vasodilation and increased platelet adhesion
Increased endothelin-1	Increased vasoconstriction
Increased plasma PAI-1	Inhibits fibrinolysis
Decreased tPA activity	Decreased fibrinolysis
Increased VWF	Increased platelet adhesion
Increased levels of IL-6	Inflammatory response
Increased levels of pro-inflammatory chemokines	Inflammatory response
Increased expression of CAMs (ICAM-1, VCAM-1, E-selectin, PECAM-1, P-selectin)	Increased binding of leukocytes and platelets. Activation of inflammatory response
Increased levels of hs-CRP	Inflammatory response
Increased cell permeability	
Increased LDL oxidation	
Increased VSMC proliferation and migration	Lesion development
Increased leukocyte adhesion	Initiation of plaque formation

(Key: NO - nitric oxide; PAI - plasminogen activator inhibitor type-1; tPA - tissue plasminogen activator; VWF - von Willebrand Factor; IL - interleukin; CAMs - cell adhesion molecules; ICAM - intercellular adhesion molecule; VCAM - vascular cell adhesion molecule; PECAM - platelet-endothelial cell adhesion molecule; CRP - C-reactive protein; LDL - low density lipoprotein; VSMCs - vascular smooth muscle cells)

Table 13: Overview of the Causes of Endothelial Dysfunction

	Cell proliferation	Apoptosis	Vasoconstriction	ROS formation	AGE formation	Permeability	Leukocyte adhesion	Platelet adhesion	CAM expression	MMP formation	NF-kB
Hyperglycaemia	+/-	+/-	+	+	+	+	+		+	+	+
Insulin resistance	+	+	+/-				+		+		+
Glycation	+	+	+	+	+	+			+		+
Oxidative Stress	+	+	+	+	+		+	+	+	+	+

(Key: ROS - reactive oxygen species; AGEs - advanced glycated end-products; CAM - cell adhesion molecule; MMP – metalloproteinase; + = for, - = against, +/- = both evidence for and against)

Table 14: Markers of Endothelial Dysfunction in Diabetes mellitus

T1DM	T2DM
Plasma VWF (except in early, uncomplicated, regulated diabetes)	Plasma VWF
VCAM-1 up-regulation	Thrombomodulin expression
Endothelin-1 expression	tPA expression
PAI-1 expression	PAI-1 expression
Collagen type IV fragments	Collagen type IV
Microalbuminuria	Microalbuminuria
Impaired NO-mediated vasodilation (except in early, uncomplicated, regulated diabetes)	

(Key: type 1 diabetes mellitus (T1DM); type 2 diabetes mellitus (T2DM); von Willebrand factor (VWF); vascular cell adhesion molecule (VCAM); tissue plasminogen activator (tPA); plasminogen activator inhibitor (PAI))

5.2. My Proposed Research

To test this hypothesis, a number of studies were planned. These included looking at the effect of high concentration of glucose and AGEs on the endothelium by investigating their effect on endothelial cell viability, cell adhesion molecule up-regulation, reactive oxygen species generation, and cytokine production. The findings from these would give an indication to whether these diabetic-like conditions directly result in abnormal endothelial function that could indicate endothelial dysfunction. They may also allow for further understanding into whether these conditions could account for the accelerated formation of lesion associated with diabetes.

5.2.1. Cell Proliferation

Cell proliferation and apoptosis are both indicators of endothelial dysfunction. They have also been reported to be increased as a result of hyperglycaemia (Curcio and Ceriello, 1992, Cagliero et al., 1995, Esposito et al., 2001). This research, however, does not look at the indirect effects of increased glucose concentrations, such as higher levels of AGEs. Therefore, I planned to investigate whether AGEs, individually or in conjunction with high concentrations of glucose, resulted in changes to cell proliferation rates.

Cell proliferation is a measure of the number of cell dividing within a sample. As such, it is closely linked indicators of the viability of the endothelium. To measure changes to endothelial cell proliferation I used a commercial cell proliferation assay as well as growth curves. For the proliferation assay a suspension of 20,000 endothelial cells were added to each well of a 96-well plate. The cells were then incubation overnight to allow for adhesion prior to treatments. Treatments consisted of a range of glucose concentrations (11, 22,

33mM), various purities and concentrations of glycated albumin, glycated haemoglobin, and a mixture of the glycated proteins with concentrations of glucose. Controls included 5.5mM glucose and non-glycated protein. To check for consistency and reproducibility, four wells were tested under each condition in three separate experiments.

The cells were then incubated for 24, 48, or 72 hours before using a commercial cell proliferation solution and a fluorescent plate reader (at 492nm) to determine viability. ANOVA and the Tukey test were used for determining statistical significance between the control and treatments.

To complement the cell proliferation studies, growth curves were to be obtained for each treatment. The growth curves were to be done by plating 24-well plates with a suspension of 10,000 cells. Each day, for 5 days or until confluent, four random areas would be counted using an inverted microscope and photographic images taken. The cell counts would then be plotted onto a graph to give a growth curve. The growth curve could then be used to compare the treatments with those of the controls to determine whether they resulted in growth inhibition, acceleration, or had no effect at all.

5.2.2. Cell Adhesion Molecule Expression

Endothelial dysfunction is associated with activation of the endothelium. Activation causes changes in the expression of cell adhesion molecules and therefore likely to result in cell adhesion. (There are a number of cell adhesion molecules that have been reported to have increased expression associated with endothelial dysfunction. An overview of these can be found in Table 15.) To investigate whether the consequence of diabetic-like conditions is endothelial activation, research was proposed to look at up-regulation of cell adhesion molecules associated with both platelet and leukocyte adhesion. The cell adhesion molecules proposed for investigation were $\alpha_v\beta_3$ (firm platelet adhesion), P-selectin (transient platelet interactions), E-selectin (leukocyte adhesion) and VWF (an intermediate associated with lots of cell adhesion molecules for both platelets and leukocytes). All these cell adhesion molecules are expressed on endothelial activation and therefore quantitative analysis was required to determine whether these conditions resulted in activation.

It was decided to look for both protein and gene expression for these cell adhesion molecules. This would provide both a check on the results obtained from one of the procedures but also allow for determining whether activation was transient or sustained.

Table 15: Cell Adhesion Molecules Involved in Endothelial Dysfunction

CAM	Detail	Reference
VCAM-1	High glucose increased expression of VCAM-1. This was inhibited by blocking PKC β	Kouroedov <i>et al.</i> 2004
VCAM-1 & ICAM-1	Incubation of HAECs with glucose increased the expression of VCAM-1 but no effect on ICAM-1 expression Incubation of HUVECs with glucose increased the expression of VCAM-1 and ICAM-1	Piga <i>et al.</i> 2007 Tsuneki <i>et al.</i> 2007
VCAM-1, E-selectin, & ICAM-1	High concentrations of glucose up-regulated the expression of these CAMs on HUVECs (tissue sample)	Altannavch <i>et al.</i> 2004
E-selectin & ICAM-1	Incubation of HSVECs with glucose increased the expression of these	Haubner <i>et al.</i> 2007
P-selectin & ICAM-1	HUVECs expressed P-selectin & ICAM-1 upon incubation with glucose	Puente Navazo <i>et al.</i> 2001
ICAM-1 & PECAM-1	Short term exposure of HUVECs to high concentrations of glucose resulted in increased levels of ICAM-1, but no effect on PECAM-1. Longer term exposure resulted in ICAM-1 up-regulation but down-regulation of PECAM-1 from that of the control	Baumgartner-Parzer <i>et al.</i> 1995
VWF	Increased expression of VWF following incubation of BAECs with HG Increased expression in diabetics and people with impaired glucose tolerance	Ascher <i>et al.</i> 2001 Leurs <i>et al.</i> 2002

(Key: CAM – cell adhesion molecule; VCAM - vascular cell adhesion molecule; HAECs - human arterial endothelial cells; PKC - protein kinase C; ICAM - intercellular adhesion molecule; HUVECs - human umbilical vein endothelial cells; HSVECs - human saphenous vein endothelial cells; PECAM - platelet-endothelial cell adhesion molecule)

5.2.2.1. Immunocytochemistry

Protein expression of the cell adhesion molecules was investigated using immunocytochemistry. Endothelial cells were cultured in 6-well plates containing coverslips. Treatments of the various concentrations of glucose and/or AGEs (as previously described) along with the relevant controls were administered when the cell were approximately 75-80% confluent (dependent on the incubation period). The cells were then incubated for either 24 or 48 hours. Subsequently, the monolayers were fixed using 70% ethanol and 4% paraformaldehyde. P-Selectin, E-Selectin, $\alpha_v\beta_3$, and VWF were labelled using primary antibodies and then a FITC conjugated secondary antibody was added. The endothelial nucleus was stained using 4',6-diamidino-2-phenylindole

(DAPI) and then a mounting medium was also applied to prevent loss of fluorescence and bleaching of fluorescein isothiocyanate (FITC).

Up-regulation of the cell adhesion molecules were investigated separately to allow for a clear representation of which ones were expressed. ADP was used as a positive control and untreated cells were used as a negative control. Non-specific binding was also investigated. All experiments were done in triplicate and the experiments were repeated three times to check for reproducibility.

The process of fixing and staining, with its many washing steps may result in changes to the endothelial cells. Therefore, I believed that it was important to compare these findings with those produced using a different method. Polymerase Chain Reaction (PCR) provides a way of investigating whether gene up-regulation occurs. It does not rely of the physical representation of the corresponding protein. It may, therefore, provide a way of looking for up-regulation prior to any expression. Also, it would allow for determining whether up-regulation is sustained over a period of time.

5.2.2.2. PCR

To determine the gene expression the cell adhesion molecules in question it was decided to use real time PCR. Primers were bought from a commercial company. For real time PCR, cultured cells (75-85% confluent T-75 flasks) were treated with various concentrations of glucose (5.5mM, 11mM, 22mM) with or without the various glycosylated proteins. Non-glycosylated proteins and 5.5mM glucose were used for negative controls. They were then incubated for either 24 or 48 hours prior to RNA extraction.

Extraction was carried out using a Total RNA Isolation Reagent (TRIR), after trypsinization of the cells. As part of this process, chloroform allows for the separation of the homogenate into two phases. RNA is contained in the aqueous phase while the DNA and proteins are in the organic phase. RNA is then precipitated by addition of isopropanol. The RNA was then incubated with RNA inhibitor and DNase prior to quantifying using a spectrophotometer. The absence of genomic material was then confirmed using gel electrophoresis.

The RNA samples were then reverse transcribed (RT-PCR) using a thermocycler. Total RNA was mixed with a RT-PCR kit and then cycled as per the procedure to obtain cDNA. This was then amplified using the supplied primers and SYBR green master mix.

Comparison of genetic and protein expression give an indication of the effect of diabetic conditions on up-regulation of these endothelial cell adhesion molecules. It was expected that there would be some up-regulation of these

cell adhesion molecules associated with diabetic-like conditions. These investigations, however, were designed to be done under static conditions unlike *in vivo*. As such, I decided that a comparison between these results and those obtained from dynamic flow conditions would give a better understanding of the processes involved in humans.

5.2.2.3. Dynamic Flow

Various papers have suggested that up-regulation of certain endothelial cell adhesion molecules can be dependent on blood flow conditions. Therefore, to explore this, dynamic flow studies were carried out to determine whether high shear rate had any effect of the expression of $\alpha_v\beta_3$, P-selectin, E-selectin, and VWF under diabetic conditions. These findings were then compared with those under static conditions.

The experimental conditions used for adhesion studies were utilized for these studies, although no platelets or leukocytes were administered. Instead, cell culture medium was pumped across the cells, with or without the various concentrations of glucose and/or AGEs (as mentioned previously). Flow conditions (physiologically normal [0.9 dyne/cm^2] and high shear rate [$1.5 \text{ \& } 3 \text{ dyne/cm}^2$]) were maintained for 24 or 48 hours prior to analysis using either immunocytochemistry or PCR.

The findings were then directly compared with those obtained without using the flow chamber. I expected that there would be greater up-regulation of the cell adhesion molecules under high shear rates but those low shear rates would be comparable with static flow. Their expression is important for leukocyte (and platelet) adhesion but, cell adhesion molecules are not the only indicator of endothelial dysfunction. Indeed, the production of reactive oxygen species is probably one of the main culprits for endothelial damage and therefore a key indication of endothelial activation.

5.2.3. ROS Production

Reactive oxygen species (ROS) is a term used to describe a number of highly reactive compounds such as free radicals, oxygen radicals and peroxides (see Table 16). They are produced as a by-product of respiration and are used for vasomotor control and for cell signalling. Their concentrations can, however, be significantly increased as a result of environmental stress. This can result in endogenous antioxidants being overwhelmed by the ROS resulting in cellular damage. This is known as oxidative stress.

Evidence suggests that hyperglycaemia increases the production of ROS (Inoguchi et al., 2000). Under normal physiological conditions the production of

superoxide anion results in hydrogen scavenging, ultimately producing water. Hyperglycaemia, however, results in an increased metabolism and hydrogen scavenging cannot keep pace.

Hyperglycaemia also results in an increase in flux through the polyol pathway (Van den Enden et al., 1995). The polyol pathway, indirectly, produces ROS. ROS are normally quenched by NADH but, because of the increase in sorbitol production, availability of NADH for ROS quenching is diminished.

Increased concentrations of peroxynitrite, superoxide anion, and nitric oxide have also been reported in diabetics (Schaeffer et al., 1999, Redondo et al., 2005, Trovati et al., 1997). Interestingly, the increase in nitric oxide does not correspond with other studies that report decreased nitric oxide synthase (NOS) production (Schaeffer et al., 1999, Queen et al., 2003, Martina et al., 1998, Rabini et al., 1998). The increase in nitric oxide is likely to indicate the initial phase of hyperglycaemia, where there is an increase in NOS and associated nitric oxide production. The decrease in NOS, however, is thought to be the result of prolonged exposure to hyperglycaemia, which is known to lead to decreased NOS and NO activity.

In diabetics, the increase in ROS production may be exacerbated by a decrease in antioxidant production. The availability of vitamin C in diabetic platelets is significantly lower than in controls (Sarji et al., 1979). As such, oxidative stress is likely to occur. Therefore, I wanted to establish whether diabetic-like conditions, *in vitro*, resulted in increased ROS production. There are, however, a number of different types of reactive oxygen species and many techniques for investigating them. All of which have their advantages and disadvantages. At present, there is no panacea for ROS detection. As such, I decided to choose a couple of methods for ROS detection that suited the available equipment. Although this is not ideal, it would allow for an indication of whether there was an increase in general ROS production under these conditions.

Table 16: Types of Reactive Oxygen Species

Name	Characteristics
Hydroxyl radical ($\cdot\text{OH}$)	Local damage; highly reactive
Superoxide anion (O_2^-)	Formed from donation of electrons from AGE formation
Hydrogen peroxide (H_2O_2)	Formed from donation of electrons from glycated proteins; not a free radical but readily forms hydroxyl radical
Hydroperoxide (ROOH)	Highly reactive
Peroxyl radical ($\text{ROO}\cdot$)	Reacts with DNA
Hypochlorous acid (HOCl)	Reacts with bio-molecules, including proteins, DNA, RNA, and cholesterol
Peroxynitrite ($\text{ONOO}\cdot$)	Formed from oxidation of nitric oxide ($\text{NO} + \text{O}_2^-$)
Nitric oxide (NO)	NOS catalyses NO production from L-arginine. Involved in vasodilation
Chlorine monoxide ($\text{ClO}\cdot$)	Highly reactive
Lipid radicals (i.e. $\text{LO}\cdot$ & LONO)	Formed by oxidation of unsaturated lipids

(Key: AGE - advanced glycated end-product; DNA - deoxyribonucleic acid; RNA - ribonucleic acid; NOS – nitric oxide synthase)

5.2.3.1. Griess Assay

Nitric oxide is a widely distributed free radical that is synthesized from L-arginine. It has a very small molecular weight and therefore can cross the endothelium and interact with the smooth muscle cells to mediate vasodilation. Free NO has a half-life of about 5 seconds and therefore direct investigations into this molecule are very difficult and not very reproducible. The Griess Assay, however, allows for detection of nitrite. (Nitrite is a stable breakdown product of NO.)

Following the usual treatments to culture cells, as detailed previously, a Griess Assay kit was used to firstly construct a standard curve for quantitation. The absorption spectrum from the culture medium for each treatment was then compared with this standard curve to determine how much nitrite was produced.

Controls were used to rule out background NO_2^- and NO_3^- and therefore restrict false positives. There are, however, some disadvantages of this method. The Griess reagent reaction is variable and therefore reproducibility can be a problem. It is also not possible, from this method, to determine how much of the sample's nitrate is converted to nitrite during analysis. The simplicity of this method and the ease of use under culture conditions, I believe, make this procedure preferable to other ways of detecting NO. NO,

however, is only one type of ROS and therefore, on its own, may not provide a true indication of endothelial function, especially because NO concentrations are dependent on NOS expression and prolonged endothelial activation can lead to reduced levels of NO. Therefore, it was decided that a more generic method for ROS analysis would also be investigated.

5.2.3.2. Dihydrorhodamine Oxidation

Dihydrorhodamine 123 (DHR) is readily oxidized by peroxynitrite to rhodamine 123, which allows for fluorescence detection. It is also oxidized directly or indirectly by, (and to greater or lesser extent by,) hydrogen peroxide, hydroxal radical, nitric oxide, superoxide anion, hyperchlorite anion, and peroxy radical. As such, it allows for a general indication of ROS concentrations rather than quantification.

Measurement of ROS concentrations was obtained by adapting a procedure used to investigate hydrogen peroxide levels in human coronary artery cells (HCECs) (Ni et al., 2004). Instead of treatments with lead and sodium acetate, the usual treatments with various concentrations of glucose and/or the different types of AGEs (and their associated controls) were administered. Detection was with flow cytometry (FL1 detection) following incubation of the endothelial cells with 2.5 $\mu\text{mol/L}$ DHR for 3 hours. Statistical analysis was by ANOVA and the Tukey test.

It has been reported that differences in experimental procedures for DHR analysis can result in discrepancies (van Pelt et al., 1996). This can make comparison with other investigations difficult. It was not seen as a barrier to this research because the results were to be compared with those from the Griess assay to give an indication rather than quantitative analysis.

The expectations were that administration of both high concentrations and AGEs would increase ROS production. Increased ROS concentration is likely to result in oxidative stress, if prolonged. This has been linked with increased cytokine production. Therefore, the presence of these signalling compounds was also investigated.

5.2.4. Cytokine Production

Cytokines are a structurally diverse group of immunomodulating agents involved in autocrine and paracrine signalling. As such, they are important in the immune response. They are produced by many types of cells, including endothelial cells, but mainly leukocytes. Cytokines are divided into lymphokines, interleukins and chemokines. Lymphokines and interleukins have a wide range of actions and are mainly produced by T-Cells, but also by monocytes and endothelial cells.

Chemokines are a specific group of cytokines that are involved in chemotaxis, leukocyte activation and migration.

Therefore, cytokines have a key role in inflammation and, as such, in atherosclerosis. Increased cytokine expression and endothelial dysfunction are associated with endothelial permeability, cell migration (monocytes, smooth muscle cells, and endothelial cells), cell proliferation and apoptosis (Dang *et al.* 2005; Shigematsu *et al.* 1999; Stehouwer *et al.* 1992). Furthermore, it is possible that they have their own part in endothelial activation / dysfunction.

To determine whether in vitro diabetic-like conditions increase endothelial cytokine production, experiments were established to detect any correlating increases in expression of IL-6, IL-8, fractalkine, and MCP-1. This would give an indication of whether this environment would increase leukocyte (and platelet) adhesion to the endothelium.

Table 17: Cytokines and Chemokines Synthesised by the Endothelium

Type	Name	Reference
Cytokines	IL-6, IL-7	(Loppnow and Libby, 1989, Roye et al., 1998)
CXC Chemokines	IL-8, ENA-78, GRO- α , IP-10	(Claise et al., 1996, Kinoshita et al., 2008, Imaizumi et al., 2004, Murakami et al., 1995, Luster and Ravetch, 1987)
CC Chemokines	Eotaxin, MCP-1	(Cushing et al., 1990, Gawaz et al., 1998, Haubner et al., 2007, Piga et al., 2007, Rothenberg et al., 1995)
CX ₃ C	Fractalkine	(Bazan et al., 1997)

(Key: IL - interleukin; ENA - epithelial-derived neutrophil attractant; GRO - growth regulated oncogene, IP - interferon- γ inducible protein; MCP - monocyte chemoattractant protein; RANTES - regulated on activation, normal T cell expressed and secreted)

Table 18: Cytokines and Chemokines Involved in Endothelial Dysfunction

Cytokine / Chemokine	Detail	Reference
IL-8	Increased production following exposure of HUVECs or HAECs to hyperglycaemic conditions	Kinoshita <i>et al.</i> 2007 (brief); Teramura <i>et al.</i> 1997 (long term)
MCP-1	Glucose induced MCP-1 expression and increased its production rate in HUVECs (T) HAECs (P)	Haubner <i>et al.</i> 2007 ; Piga <i>et al.</i> 2007; Takaiski <i>et al.</i> 2003

(Key: TF - tissue factor; tPA - tissue plasminogen activator; PAI-1 - plasminogen activator inhibitor type-1; HG - high glucose; HAECs - human arterial endothelial cells; VSMCs - vascular smooth muscle cells; IL - interleukin; HUVECs - human umbilical endothelial cells; MCP - monocyte chemotactic protein)

5.2.4.1. ELISA

To determine cytokine up-regulation in diabetic-like conditions, enzyme-linked immunosorbent assay (ELISA) was planned. ELISA is a highly selective technique that allows for quantification (via the use of standard curves) of soluble cytokines found in the culture supernatants.

To avoid the need for serial dilutions to create a standard curve for quantification of the cytokines detected, I decided that a commercial sandwich cytokine ELISA kit for IL-6, IL-8, Fractalkine, and MCP-1 (monocyte chemotactic protein) would be used. The kit protocol would be followed to compare the optical density of the controls alongside of those for high glucose and/or AGE incubations. Statistical significance would be determined using AVOVA and the Tukey test.

The assumption was made that the cytokines originated from the endothelial monolayer, because these would be the only cells present in these studies. To check this, it was decided that these findings would be compared with flow cytometry studies.

5.2.4.2. Flow Cytometry

Flow cytometry allowed for detection of endothelial bound cytokines. The endothelial cells were prepared and treated, as detailed previously. Following detachment by trypsin they were fixed and permeabilised using a commercial cell fixing solution, and then the cytokines were labelled using FITC-conjugated antibodies. Each cytokine was analysed separately to reduce interference, as well as reduce cost (FITC-labelled antibodies tend to be cheaper than those detected by other filters) and allow for experimental simplicity by not needing to compensate for fluorescence spectral overlap.

It was expected that comparison of the density plots for IL-6, IL-8, Fractalkine, and MCP-1 would indicate that diabetic-like conditions increased cytokine production in a time dependent fashion. I did, however, expect to find that this was more pronounced in the ELISA studies and would be further exacerbated by high shear flow rates.

5.2.4.3. Dynamic Flow

Shear stress is known to lead to opening of the calcium-activated and potassium-activated channels. The resulting flux causes hyperpolarisation of the endothelial cells and activation of transcription factors, such as NF- κ B. This transcription factor is known to induce cytokine and endothelial nitric oxide synthase (eNOS) expression. Therefore, it is likely that there is an associated increase in cytokine production as well as cell adhesion molecule up-regulation and nitric oxide production.

As such, both the ELISA and flow cytometry studies were repeated under flow conditions, as detailed previously. For ELISA, the supernatant was collected over the period of the treatments to determine total concentrations. The flow cytometry experiments were done at specific incubation times (0, 24 and 48 hours). This allowed for assessment of whether high shear flow rates increased cytokine production under diabetic-like conditions.

All of these experiments would allow for a more encompassing view of how high glucose and AGEs affected endothelial activation and dysfunction. From this, it could be determined whether one of these environments, or a combination of the two had the greatest effect on normal endothelial function. This would give insight into what causes accelerated atherosclerosis in diabetics. Insight would also be gained, by comparison with the adhesion studies, into whether platelet adhesion or interaction with the endothelium is an initiator of lesion formation in these conditions.

5.3. Research to Date

There has been a wealth of studies investigating endothelium dysfunction. It is still, however, not known what initiates it. Endothelial dysfunction encompasses a number of changes to the vessel wall, including changes in ROS production, cell adhesion molecule up-regulation, and vascular tone. It is therefore difficult to determine what the initiating factor is. All these changes impact on each other and it is likely that circumstances dictate the order of events. Studies that have involved investigating the effect of diabetic conditions on the endothelium are reviewed here.

5.3.1. Cell Viability and Proliferation

One indicator of endothelial dysfunction is abnormal cell proliferation. There have been a number of in vitro studies that have found that high glucose incubation resulted in a decreased cell count (Han et al., 2005, Ascher et al., 2001, Lorenzi et al., 1985, Varma et al., 2005) and reduced cell proliferation (Curcio and Ceriello, 1992). An increase in endothelial apoptosis has also been reported (Sheu et al., 2005, Chai et al., 2000, Piconi et al., 2006, Quagliaro et al., 2007, Baumgartner-Parzer et al., 1995, Tsuneki et al., 2007). It was found that by treating HUVECs with stable or oscillating high concentrations of glucose resulted in ROS mediated apoptosis (Piconi et al., 2006). This suggests that it is possible that hyperglycaemia, in vivo at least, may trigger endothelial apoptosis and therefore leukocyte and platelet adhesion.

Investigations into cell viability, the proportion of living cells, are not so clear cut. It would appear that different types of glucose can affect viability. In one study, decreased HUVEC count was not associated with cell viability (the number of living cells was measured by excluding Trypan blue stained cells) when the cells were incubated with L-glucose (Varma et al., 2005). Others have reported that D-glucose decreased cell viability (Trypan blue staining) of human arterial endothelial cells (HAECs) as well as HUVECs, therefore suggesting that it is not cell dependent (Morishita et al., 1997, Rojas et al., 2003). With D-glucose being the sugar available in vivo, it is likely that these studies give a more correct indication of the effect of hyperglycaemia on cell growth and viability.

This research suggests that in vitro induced hyperglycaemia reduces cell proliferation and viability, although viability is dependent on the type of sugar. A number of studies have investigated the effect of AGEs on cultured endothelial cells. A 40% reduction in cell proliferation has been reported as a consequence of treatments with glycated foetal serum albumin (AGE-FSA) (Ruggiero-Lopez et al., 1997). Treatment with glycated bovine serum albumin (AGE-BSA), however, resulted in an increase in cell numbers of 156%. Other studies have also reported increased cell proliferation following incubations with AGE-BSA (Hoffmann et al., 2002, Chibber et al., 1997). These studies have used relatively high concentrations of glycated albumin. Lower concentrations of AGE-BSA appear to reduce cell proliferation and increases endothelial apoptosis (Chibber et al., 1997, Kowluru, 2005). With concentrations of glycated proteins increasing with age and exposure to increased sugar concentrations, it is likely that lower concentrations are more representative of initial lesion formation. As such, cell repair is likely to be diminished. This may, however, be the result of the endothelial cells not initiating cell repair quickly under these conditions and that extended periods of exposure may result in increased proliferation. This theory was therefore explored with the research proposed in this thesis.

It is clear that diabetic-like conditions have an effect on cell proliferation and

viability. However, it would appear that type and concentration of sugar and AGEs play a significant role in this. As such, is this also the case for expression of cell adhesion molecules? This is explored in the next section along with general research into the effect of diabetic conditions on cell adhesion molecule expression.

Table 19: Endothelial Integrity

Detail	Reference
Permeability & migration	<p>HG increased cell migration (Stehouwer et al., 1997)</p> <p>Increased permeability linked with metal ions in PBS (used to prepare the AGEs) not the AGEs (Hui et al., 2001)</p> <p>HG induced changes to actin-binding proteins and focal adhesion proteins (Lee et al., 2004)</p> <p>Increased permeability is associated with PKC (Dang et al., 2005)</p> <p>HG increased albumin permeability in non-diabetic rats (Scalia et al., 2007)</p>
Cell number (proliferation & apoptosis)	<p>HG induces decreased HUVEC cell count (Han et al., 2005, Lorenzi et al., 1985)</p> <p>HG induces decreased HUVEC cell count (probably by apoptosis and not decreased cell viability) (Varma et al., 2005)</p> <p>Incubation of HAECs and VSMCs with high concentrations of D-glucose decreased cell viability, but L-Glucose and mannitol did not (Morishita et al., 1997)</p> <p>Cell proliferation is reduced following HG culture (Curcio and Ceriello, 1992)</p> <p>Decreased BAECs cell count following incubation with HG (Ascher et al., 2001)</p> <p>HUVECs incubated with HG underwent apoptosis (Piconi et al., 2006, Quagliaro et al., 2007, Sheu et al., 2005, Tsuneki et al., 2007, Baumgartner-Parzer et al., 1995)</p> <p>HG up-regulated COX-2 expression via NF-kB, this lead to HUVEC apoptosis via caspase-3 activation. A combination of HG, high insulin, and ox-LDL resulted in greater apoptosis than each one alone (Chai et al., 2000)</p> <p>AGE-FSA decreased while AGE-BSA increased cell proliferation (Ruggiero-Lopez et al., 1997)</p> <p>AGE-BSA increased cell proliferation (Hoffmann et al., 2002)</p> <p>Low concentrations of AGE-BSA decreased while high concentration increased cell proliferation (Chibber et al., 1997)</p> <p>AGE-BSA increased bovine retinal EC apoptosis (Kowluru, 2005)</p>

(Key for Table 19: HG - high glucose; HUVECs - human umbilical vein endothelial cells; PKC - protein kinase C; PBS - phosphate buffered saline; AGEs - advanced glycation end-products; HAECs - human aortic endothelial cells; VSMCs - vascular smooth muscle cells; BAECs - bovine aortic endothelial cells; PI - phosphatidyl inositol; LDL - low density lipoproteins); ECs – endothelial cells

5.3.2. Cell Adhesion Molecule Expression

The up-regulation of cell adhesion molecules may well be a marker of endothelial dysfunction (Liuba et al., 2003). It is known that activation of the endothelium initiates or increases the expression of numerous adhesion receptors and therefore it is likely to be a key determinant in endothelial dysfunction, as well as initiating atherosclerosis. Investigation into expression of adhesion molecules via protein and gene analysis, as well as immunocytochemistry and blocking antibodies, should indicate whether adhesion molecules for leukocytes and platelets are present in diabetic conditions.

There is evidence to suggest that hyperglycaemia increases the expression of certain endothelial receptors (Table 15). It is generally recognised that the levels of soluble adhesion molecules should give an indication of expression and are known to be a good indicator of endothelial dysfunction (Serebruany and Gurbel, 1999, Peng et al., 2005, Barbaux et al., 2001, Xie et al., 2000). Khare and colleagues, however, found that there was no correlation between soluble cell adhesion molecules and endothelial damage in patients with cardiovascular disease (Khare et al., 2005). This was based on 120 patients that had stabilized within 8-10 weeks following a myocardial infarction. Patients with diabetes and hypertension were ruled out. Family members without visible signs of cardiovascular disease as well as non-related controls were used to compare levels of soluble levels of cell adhesion molecules (sP-selectin, sE-selectin and sPECAM-1) using immunoassays. The results may well have been because there was a decreased clearance rate of adhesion molecules present in the plasma, rather than increased production. Or they may have been related to advanced stages of atherosclerosis and therefore might not represent levels of soluble adhesion molecules in the early stages of atherosclerosis.

When looking at the plasma levels of adhesion molecules and ligands it is also important to note that some are produced by more than one cell type, for instance VWF is synthesised by platelets as well as endothelial cells. With cell culture studies this is not a problem but is a key factor for in vivo research. When looking at patient studies of adhesion molecule it is also difficult to determine the originating cause of up-regulation. For instance, when looking at diabetics, to understand whether hyperglycaemia increases expression of both soluble and cell adhesion molecules, hypertension and hypercholesterolaemia need to be considered. Research has suggested that both of these risk factors for diabetes are linked the up-regulation of soluble adhesion molecules (Lim et al., 2004, Steiner

et al., 1994).

Cell adhesion studies therefore allow for determining what effect hyperglycaemic conditions have to cell adhesion molecule expression. Research has indicated that vascular cell adhesion molecule-1 (VCAM-1) expression is increased following incubation of endothelial cell monolayers with high concentrations of glucose. This is thought to be a protein kinase C (PKC)-dependent mechanism because inhibition of PKC β inhibited glucose induced up-regulation of VCAM-1 (Altannavch et al., 2004, Kouroedov et al., 2004, Piga et al., 2007, Tsuneki et al., 2007).

Altannavch and colleagues not only found increased expression of VCAM-1, they also noted that E-selectin and ICAM-1 were also up-regulated under these conditions (Altannavch et al., 2004). Further investigations support the expression of E-selectin, suggesting that accelerated atherosclerosis in diabetes could be the result of E-Selectin dependent leukocyte adhesion (Haubner et al., 2007).

Other cell adhesion molecules have been found to be up-regulated under hyperglycaemic conditions. There was increased expression of P-selectin and von Willebrand factor (VWF) in endothelial cells incubated with high concentrations of glucose (Ascher et al., 2001, Puente Navazo et al., 2001). Raised levels of VWF have also been reported in diabetics with insulin intolerance (Baumgartner-Parzer et al., 1995). This suggests that VWF could mediate platelet and leukocyte adhesion in diabetics.

Platelet-endothelial cell adhesion molecule-1 (PECAM-1), on the other hand, does not appear to have a major role in adhesion to endothelial cells. It has been reported that there was no change in PECAM-1 expression following short-term incubation with glucose, but long-term exposure resulted in down-regulation of this receptor (Baumgartner-Parzer et al., 1995). There does not, however, appear to be any other evidence to support or contradict this research.

Hyperglycaemia increases VCAM-1, ICAM-1, E-Selectin, P-Selectin and VWF expression but possibly not PECAM-1. This would indicate that leukocyte and possibly platelet adhesion is likely to be increased under these conditions. Obviously, extended periods of hyperglycaemia is also associated with increased AGE concentration. Indeed, leukocyte rolling has been reported to be significantly increased following endothelial incubation with AGEs but reduced by blocking E-Selectin, ICAM-1, and VCAM-1. This would suggest that both high glucose and AGEs increase endothelial cell adhesion molecule expression.

Rabbit studies have shown that daily intravenous injections of low concentrations (16mg/kg/day) of glycated rabbit serum albumin (AGE-RSA) led to increased

expression of VCAM-1 and ICAM-1 but not E-Selectin, following 4 months of treatment (Vlassara et al., 1995). This was observed at areas where lesions had formed. Expression, determined by monoclonal antibodies, was more pronounced in animals that were also fed on a cholesterol-rich diet.

Cell culture studies show contradictions in the cell adhesion molecules up-regulated, see Table 20. There are reports that both PECAM-1 and ICAM-1 have increased expression in some reports and not in others (Meng and Liu, 2003, Negrean et al., 2007, Otero et al., 2001, Schmidt et al., 1995, Sengoelge et al., 1998, Mamputu and Renier, 2004). The difference in expression of PECAM-1 appears to be related to the treatments. Incubations with glycated bovine serum albumin (AGE-BSA) had no effect, while glycated fibrinogen (AGE-Fibrinogen) increased both transcriptional as well as surface expression.

As mentioned previously, ICAM-1 expression has been reported to be increased in rabbits injected with AGE-RSA (Vlassara et al., 1995). It would appear, however, that the concentration of glycated protein used can affect expression of this adhesion molecule. Incubations with AGE-BSA and AGE-Fibrinogen have been reported to both increase expression and have no significant effect (Schmidt et al., 1995, Sengoelge et al., 1998, Mamputu and Renier, 2004). The studies that showed increased expression used higher concentrations of AGE-BSA than the other. It should be noted, however, that the presence of endotoxins could have been involved. These studies either do not mention whether precautions were taken to minimize their presence or reported that small levels were present and therefore they may have contributed to ICAM-1 up-regulation.

VCAM-1 is reported to be increased following incubations with both AGE-BSA and AGE-Fibrinogen. There have, however, been some discrepancies relating to methodologies. It has been suggested that the presence of endotoxins in the preparations can affect VCAM-1 up-regulation. Valencia and colleagues treated human microvascular endothelial cells (HMECs) with AGE-BSA for 18 hours (Valencia et al., 2004). (It should be noted that they found a difference in VCAM-1 expression based on the sugar used for BSA glycation.) Kunt and team, however, incubated human umbilical vein endothelial cells (HUVECs) with 1 μ M of AGE-BSA for 5 hours (Kunt et al., 1999). Therefore, the differences may well be as a consequence of experimental differences, possibly cell origin, concentration, (it was not possible to determine the molar concentration of AGE-BSA administered in the paper of Valencia et al.,) or exposure time.

Treatment with an anti-oxidant, α -lipoic acid, reduced VCAM-1 expression (Kunt et al., 1999). Antioxidant co-incubations were also found to decreased ICAM-1 expression (Mamputu and Renier, 2004). This would suggest that both ROS as well as cell adhesion molecules closely interact and therefore are difficult to investigate in isolation. Therefore, we can establish from the literature that it is

likely that hyperglycaemia and increased concentrations of AGEs increase the expression of some endothelial cell adhesion molecules. Treatments with antioxidants indicate that the expression of these cell adhesion molecules can be modulated by ROS concentrations. As such, the information available on the effect of diabetic-like conditions on ROS has been investigated here.

Table 20: Effect of AGEs on Cell Adhesion Molecule Expression

Model	CAM Expression	Reference
Rabbit	VCAM-1&ICAM-1 but not E-Selectin expression was increased following AGE-RSA injections for 4 months	(Vlassara et al., 1995)
HUVECs	No effect on PECAM-1 expression following incubations with AGE-BSA	(Meng and Liu, 2003, Otero et al., 2001)
HUVECs	Increased expression of VCAM-1 but not ICAM-1 following incubations with AGE-BSA	(Schmidt et al., 1995)
HUVECs	Treatments with AGE-Fn & AGE-Alb. Increased transcription levels of E-Selectin at 2.5 hours but not at 10 hours. No increase in surface expression. Increased surface expression of ICAM-1 & VCAM-1. Increased surface and transcriptional expression of PECAM-1	(Sengoelge et al., 1998)
HUVECs	Increased VCAM-1 expression following treatment with endotoxin-free AGE-BSA. This was reduced by pre-treatment with α -lipoic acid	(Kunt et al., 1999)
HMECs	AGE-BSA increased VCAM-1 expression unless it was endotoxin-free	(Valencia et al., 2004)
BRECs	AGE-BSA (low endotoxin) treatments increased ICAM-1 expression. This was reduced by treatment with antioxidants	(Mamputu and Renier, 2004)

(Key: VCAM – vascular cell adhesion molecule; ICAM – intracellular adhesion molecule; AGE – advanced glycation endproduct; RSA – rabbit serum albumin; HUVECs – human umbilical endothelial cells; PECAM – platelet-endothelial cell adhesion molecule; BSA – bovine serum albumin; Fn – fibronectin; Alb – albumin; HMECs – human microvascular endothelial cells; BRECs – bovine retinal endothelial cells)

5.3.3. Reactive Oxygen Species Production

Oxidative stress has been linked with diabetes (Aydin et al., 2001, Leoncini et al., 1997). This phenomenon occurs when the concentration of ROS outnumber those of antioxidants. The result can be endothelial dysfunction. This, in turn, sets off a series of events, including up-regulation of cell adhesion molecules (as mentioned previously), prostanoid production, and changes in vasomotor status.

Hyperglycaemia has been linked with the production of ROS. It has been suggested that high glucose concentrations increase ROS production 1.5-fold (Graier et al., 1999). High glucose concentrations have also been shown to reduce antioxidant binding affinity (Ülker et al., 2004, Weidig et al., 2004, Kunisaki et al., 1993). As such, it would appear that there is not only an increase in ROS formation but that there is also a decrease in the ability to counter them.

There are a number of different ways of detecting ROS. All of which have their advantages and disadvantages. Most concentrate on specific ROS but some are more general. There is not, however, one method that can be used successfully to give the whole picture. As such, a lot of research appears to concentrate on one ROS. Superoxide anion levels, for instance, has been used quite substantially. The research indicates that this ROS is elevated following incubation of various endothelial cell lines with high concentrations of glucose (Cosentino et al., 1997, Graier et al., 1999, Tsuneki et al., 2007, Inoguchi et al., 2000). There may, however, be some time-dependent factors.

It has been reported that, initially, hyperglycaemia resulted in an increase in gene and protein expression of eNOS (Tsuneki et al., 2007, Srinivasan et al., 2004a, Ho et al., 1999). This resulted in an increase in production of the main vasodilator, nitric oxide, resulting in vasodilation and increased blood flow (Sobrevia et al., 1996, Kasai et al., 2001, Tesfamariam and Cohen, 1992).

Long-term hyperglycaemia, on the other hand, results in a decrease in the concentration or reduction of the efficiency of eNOS (Cosentino et al., 1997, Noyman et al., 2002, Quagliaro et al., 2007, Kasai et al., 2001, Pieper et al., 1996, Srinivasan et al., 2004a). Therefore, although there is an increase in the production of eNOS, there is a decrease in nitric oxide due to the inefficiency of eNOS in producing nitric oxide. Decreased nitric oxide is coupled with increased endothelin-1 to result in vasoconstriction (Keynan et al., 2004, Park et al., 2000, Yamauchi et al., 1990).

There may well be a cell-dependent response. It has been reported that coronary microvascular endothelial cells are unaffected, in relation to nitric oxide and eNOS (Weidig et al., 2004). The experiments were carried out using similar concentrations of glucose to others (22mmol/L) and incubation was for 7 days. All the other investigations have used endothelial cells from either arteries or veins and therefore this appears to account for this difference. It, however, may just be the result of culture conditions – for instance, passage number, infrequency of medium replenishment and infection.

The availability of nitric oxide has an impact on vasomotor control, in that it is a vasodilator. Therefore, initial high glucose concentration will increase vascular dilation but with continued exposure vasoconstriction will occur. Added to this, research has demonstrated that there is impairment in the production and response of the endothelium to vasodilatory stimuli and an increase in the production of vasoconstrictors in diabetic patients, even in the absence of pathological complications (Title et al., 2000).

Other ROS have been reported to be increased in hyperglycaemic conditions, see

Table 21. There is not a lot of research, but what there is suggests that both superoxide anion and peroxide are increased following exposure to high concentrations of glucose. It should be noted, however, that peroxide expression was only increased following 48 hours of exposure and superoxide anion was only reported following exposure for 5 days.

ROS are also known to stimulate production of prostanoids. Prostanoids are prostaglandins, prostacyclins, and thromboxanes, and include the vasoconstrictor thromboxane A₂ and the vasodilator PGI₂. Hyperglycaemia induced in tissue from animal diabetic models was reported to exhibit increased levels of prostanoids, possibly as a result of synthesis of ROS (Tesfamariam et al., 1990, Tesfamariam and Cohen, 1992, Shirahase et al., 1987). Blocking prostacyclin and thromboxane receptors was shown to induce vasodilation. Inhibition of prostacyclin production on its own, however, had no effect (De Vriese *et al.* 1999). Other studies have also found that increased thromboxane and decreased prostacyclin production accounts for vasoconstriction under conditions of high glucose (Cosentino et al., 2003, Sobrevia et al., 1996).

Therefore, hyperglycaemia appears to result in time-dependent changes to the expression of ROS. Nitric oxide, a potent vasodilator, is increased on initial exposure to high glucose concentrations but is reduced after medium to long term exposure. Peroxide and superoxide anion production are likely to be only expressed following non-intermittent exposure. Whether the presence of AGEs also alters ROS availability is explored.

There does not, however, appear to be much published research indicating the effect of AGEs on endothelial ROS production, other than relating to nitric oxide. A decrease in both nitric oxide and eNOS has been reported following AGE exposure (Xu et al., 2003, Hogan et al., 1992, Rashid et al., 2004, Rojas et al., 2000). This correlates with the effect of hyperglycaemia on nitric oxide and eNOS production. Interestingly, it has also been reported that nitric oxide decreases both AGE formation and AGE-dependent leukocyte adhesion (Asahi et al., 2000, Xiang et al., 2006). This would, again, link ROS with cell adhesion molecules and associated leukocyte adhesion. Whether or not this allowed for increased platelet adhesion has been addressed in this proposed research.

Overall, it would appear that diabetic conditions (both hyperglycaemia and AGEs) increase the production of some ROS, notably peroxide and superoxide anion. Nitric oxide, however, is decreased upon medium to long term exposure. This, along with increased vasoconstrictor production, causes vasoconstriction. This leads to an increase in the shear rate; rates greater than 70 dyn/cm² are thought to cause endothelial erosion, platelet adhesion, and plaque rupture, however, atherosclerotic plaque fissures only develop in areas where the shear rate is low (< 6 dyn/cm²) (Esper *et al.* 2006). As such, the increase in nitric oxide

may provide conditions more suitable for lesion formation.

Table 21: Reactive Oxygen Species Production by Endothelial Cells under Diabetic Conditions

Compound	Detail	Reference
ROS	Increased ROS concentrations following endothelial incubation with HG	(Inoguchi et al., 2000, Tsuneki et al., 2007)
NO	Increased	Trovati <i>et al.</i> 1997
NOS	Increased in HUVECs treated with HG Initial increase. Reduced levels after 24 hours of HUVEC incubation with HG	(Tsuneki et al., 2007) (Ho et al., 1999)
NO & NOS	Decreased levels of NO but increased levels of NOS in HAECs treated with HG No effect on expression of either in CMECs treated with HG Short term exposure caused an increase while long term reduced expression Decreased expression following treatment of HUVECs with AGEs	(Cosentino et al., 1997) (Weidig et al., 2004) (Srinivasan et al., 2004a) (Xu et al., 2003, Hogan et al., 1992, Rashid et al., 2004, Rojas et al., 2000)
NO, NOS & SO	Increased levels in HAECs treated with HG	(Cosentino et al., 2003)
SO	Increased	(Graier et al., 1999)
H ₂ O ₂	Increased expression following 48 hours of HUVEC incubation with HG	(Ho et al., 1999)

(Key: ROS - reactive oxygen species; HG – high glucose; HUVECs – human umbilical endothelial cells; NO - nitric oxide; NOS - nitric oxide synthase; HAECs – human artery endothelial cells; CMECs – coronary microvascular endothelial cells; AGEs – advanced glyated endproducts; SO - superoxide anion)

5.3.4. Cytokine Expression

ROS production can also be increased by production of cytokines (Yang et al., 2007). It has also been reported that oxidative stress can stimulate production of certain cytokines, including IL-8 (Vlahopoulos et al., 1999). There has, however, been very little breadth to investigation into the effect of diabetic-like conditions on cytokine production by the endothelium.

There has, however, been a reasonable amount of research looking into the effect of high concentrations of glucose on IL-6, IL-8 and MCP-1 expression in cultured endothelial cells. Increased expressions of IL-6, IL-8 and MCP-1 have been reported (Piconi et al., 2004, Srinivasan et al., 2003, Temaru et al., 1997, Kinoshita et al., 2008, Piga et al., 2007, Takaishi et al., 2003, Srinivasan et al., 2004c, Srinivasan et al., 2004b). It is interesting to note that in one of these studies it was reported that intermittent incubations of high glucose significantly increased IL-6 production above that of stable high glucose (Piconi et al., 2004).

The in vitro research correlates with increased levels of IL-6 and MCP-1 in diabetics (Gomez et al., 2008). There are, however, some discrepancies when these studies are compared endothelial cells obtained from diabetics. Haubner and colleagues only reported increased levels of MCP-1, not IL-6 or IL-8 in saphenous vein endothelial cells removed during a coronary artery bypass (Gomez et al., 2008, Haubner et al., 2007). The differences are likely to be as a result of the diabetics having advanced vascular disease. (Those use for the in vivo studies had no associated complications.)

Overall, however, it would appear that it is likely that hyperglycaemia, whether short-term, long-term, or intermittent results in increased production of cytokines in vitro. The increased expression of both IL-6 and IL-8 has also been linked with increased monocyte adhesion to the endothelium.

There does not appear to be any studies that have investigated the effect of AGEs on cytokine production by endothelial cells. There have, however, been some that have looked at IL-8 and MCP-1 expression by human corneal keratocytes and mesangial cells (Bian et al., 1998, Yamagishi et al., 2002). These found an increase in production following incubation with various AGEs. It is therefore possible that these conditions would also induce production of these cytokines by endothelial cells, therefore initiating monocyte adhesion and activation. Indeed, the research proposed within this thesis proposed to address this gap in the research and allow for determining whether diabetic-like conditions induce changes to the endothelium that account for accelerated atherosclerosis.

Table 22: Cytokine Production by Endothelial Cells under Diabetic Conditions

Cytokine	Detail	Reference
IL-6	Increased expression under hyperglycaemic conditions Increased in diabetics Unchanged in diabetic ECs	(Srinivasan et al., 2004b, Piconi et al., 2004) (Gomez et al., 2008) (Haubner et al., 2007)
IL-8	Increased expression under hyperglycaemic conditions Unchanged in diabetic ECs	(Kinoshita et al., 2008, Srinivasan et al., 2004c, Srinivasan et al., 2003, Temaru et al., 1997) (Haubner et al., 2007)
MCP-1	Increased expression under hyperglycaemic conditions Increased in diabetics	(Piga et al., 2007, Takaishi et al., 2003) (Gomez et al., 2008, Haubner et al., 2007)

(Key: IL – interleukin; ECs – endothelial cells; MCP – monocyte chemotactic protein)

5.4.What can be concluded?

Diabetes is associated with oxidative stress, endothelial dysfunction and accelerated atherosclerosis. Both oxidative stress and endothelial dysfunction are closely related conditions, in that they are both associated with increased ROS production and cell adhesion molecule up-regulation. Oxidative stress occurs when ROS production overwhelms that of antioxidants. Endothelial dysfunction is not clearly defined, but it is associated with abnormal endothelial function (including activation, cell adhesion molecule up-regulation, ROS and cytokine production, as well as decreased cell viability). As such, they are possible contenders for accelerated atherosclerosis under diabetic conditions.

Hyperglycaemia has been reported to have a wide array of effects upon the endothelium. It has been linked with decreased cell viability (potentially as a result of apoptosis), up-regulation of cell adhesion molecules, as well as increased production of ROS and cytokines. Up-regulation of a number of cell adhesion molecules has been reported, including VCAM-1, E-selectin, ICAM-1, P-selectin, and VWF. It also increases the production of pro-inflammatory and pro-thrombogenic cytokines, including IL-6, IL-8 and MCP-1. However, it would appear that diabetic endothelium from patients with advanced stages of atherosclerosis only exhibit heightened levels of MCP-1. There did not appear to be any published research that investigated the effect on other endothelial cytokines, including IL-7, ENA-78, GRO- α , IP-10, eotaxin, and fractalkine. This appears to be strange, especially when fractalkine has been associated with inflammatory diseases such as atherosclerosis.

AGEs have also been reported to decrease cell viability, cell adhesion molecule up-regulation, as well as ROS production. There do, however, appear to be differences dependent on the type of protein glycated, the type of sugar, the preparation, and concentration of the glycated protein used; there is also no information on cytokine production. The research that has been done generally gives similar results to those reported for hyperglycaemia – reduced cell viability, up-regulation of VCAM-1, ICAM-1, and production of IL-6, IL-8 and MCP-1.

These are, therefore, likely to indicate that the endothelium behaves in an abnormal fashion after exposure to high concentration of glucose and AGEs. The reports would correlate with the endothelium being dysfunctional. There is still however, some aspects that have not been fully covered in the literature. Most of the research has been in vitro. There are a number of discrepancies in the findings. Various reasons can be proposed for this, including glucose preparations and the cell line, but it is clear that more research is needed to gain a clearer understanding of the effect of hyperglycaemia on the endothelium.

To continue these investigations on the different aspects of endothelial dysfunction in diabetic-like conditions, a number of studies were proposed. They incorporated general experiments, such as cell viability (cell proliferation and viability studies), cell

adhesion molecule up-regulation, ROS production, as well as cytokine expression. Expansion of current studies was part of this and included analysis of a range of cytokines, not just IL-6, IL-8 and MCP-1, various concentrations of glucose and different glycated proteins. The aim was to fill in some of the gaps in the literature and, by doing so, provide a better understanding of whether hyperglycaemia and AGEs could account for accelerated lesion formation in diabetics. This research would also allow for a greater understanding of whether platelet adhesion/interaction with the endothelium could initiate atherosclerosis via endothelial dysfunction.

With only in vitro experiments possible for this research, further investigations would be required. Although, they provide a good understanding of isolated effects, in humans there are many more factors involved. These cannot be covered in laboratory based investigations and are difficult to interpret. With new techniques becoming available, it would be important to build on this work to gain a clearer understanding of how everything pieces together.

6. Conclusion

Atherosclerosis is characterized by fatty deposits, known as foam cells, within the vascular wall. Over time, these deposits build up to form more complex lesions that consist of a lipid core and a cap, which leads to partial occlusion of the blood vessel. The core contains lipid laden macrophages along with cellular debris, free lipids and cholesterol. The cap is made up of layers of smooth muscle and endothelial cell. This can become calcified and vasculated, making it brittle and liable to fissuring. As such, these more advanced lesions are often associated with causing conditions such as myocardial infarction, strokes and claudication. These result from fissuring of the lesion cap, exposure of cell adhesion molecules and cytokines that attract platelets and therefore thrombus formation and occlusion of the blood vessel.

There are a number of human conditions associated with accelerated rates of lesion formation. These include diabetes, systematic lupus erythematosus and arthritis. These diseases all involve an immune response. There are, however, a number of immunological conditions that are not linked to accelerated atherosclerosis, such as coeliac disease and asthma. There are also some non-immunological conditions associated with accelerated atherosclerosis, including smoking and radiation.

The processes involved in initiating atherosclerosis are not fully understood at present. There have been a number of hypotheses published including that of Ross and Glomerset who suggested that atherosclerosis may result from injury to the endothelium resulting in denudation. This has been built on by others including the response to injury theory that suggested that injury did not cause denudation but, instead, resulted in functional changes to the endothelial cells. Suggested changes included ROS and cytokine production as well as cell adhesion molecule upregulation. Recently there have been a number of papers that have suggested that platelets may be involved. As such, this thesis proposed to investigate whether increased platelet interaction with the endothelium could hold the key. In doing so, it aimed to gain a better understanding of the processes involved in both atherogenesis and endothelial dysfunction. Further research could then be done to target foam cell production, providing a preventative treatment strategy. This would reduce the suffering of many patients and also reduce the massive health care costs associated with treatments linked to atherosclerosis.

For platelets to be involved in atherogenesis they must be some sort of interaction with the endothelium. It was initially thought that platelet adhesion only occurred following endothelial denudation, it is now clear that this is not the case. There is a wealth of information to suggest that platelets can adhere to the endothelium, under various conditions.

It is now generally accepted that platelet-endothelial interactions play a key role in acute coronary events associated with advanced atherosclerosis, such as myocardial infarction, but there is some evidence to suggest that they are also involved in initiating plaque formation. Over the last 10 years, however, a number of papers have been published

suggesting that platelet adhesion may initiate lesion formation. These include studies that have shown that monocyte transmigration is accelerated by platelets. It has also been suggested that platelets are involved because monocytes do not have receptors for oxLDL and therefore platelets, which do, are a prerequisite for foam cell formation.

The majority of research investigating lesion formation has not reported platelet adhesion. There are, however, some papers that have found platelets adhered prior to lesion formation. This has been reported in humans (15-34 year olds that had no visible signs of cardiovascular disease), Apo E^{-/-} mice and rabbits. There is also a number of studies that have shown that there is increased lesion formation when platelets are present than when there are only leukocytes. It does not, however, conclusively show that platelets are involved in atherogenesis because it was not possible to definitively say what happened to these platelets. Indeed, without carrying specific studies to investigate this it is difficult to conclusively show cause and effect.

There have been a number of studies that have investigated the cell adhesion molecules involved in these platelet interactions with the endothelium by using monoclonal antibodies. There are some discrepancies based on the type of cells and antibody used. It is, however, possible to conclude that endothelial and GPIb, and platelet GPIIb/IIIa, as well as VWF and fibrinogen are involved. The extent of inhibition created by using blocking studies was dependent on the type of interaction investigated, for instance, different adhesion molecules are involved in transient attachments and firm adhesion.

Inhibition of platelet binding has also been investigated by looking at anti-platelet drugs. The use of anti-platelet drugs suggests that platelets may well be involved in atherogenesis. Administration of many of these drugs has resulted in reduced lesion coverage and few layers / intimal thickness. These include low levels of aspirin, S18886, ticlopidine, clopidogrel, celecoxib, cilostazol, dipyridamole, and sulfinpyrazone. A cocktail of anti-platelet drugs appears to have a greater effect on reducing lesion formation compared to administration of only one. This would suggest that there are numerous ways that platelets could be involved, for instance aspirin inhibits platelet activation while clopidogrel, ticlopidine, cilostazol and S18886 inhibit platelet aggregation. Aspirin also inhibits endothelial prostacyclin synthesis and availability of oxLDL. As a consequence, it has been suggested that a reduction in lesion formation associated with treatment with aspirin may be as a result of its anti-inflammatory and antioxidant properties. This is potentially supported by the report of celecoxib treatment significantly reducing lesion formation.

Conditions associated with platelet abnormalities, such as von Willebrand disease, Glanzmann Thrombasthenia, and Bernard-Soulier disease have been shown to develop atherosclerosis. Deficiencies in VWF in mice and pigs, however, suggest that adhesion mechanisms may be involved in lesion formation. This might be because the process of atherogenesis is different between species, but there are a number of other factors that may contribute to the differences, including differences in methodology. Humans were

often treated for bleeding episodes, but pigs would not be. Patients with type 3 disease were given clotting factors and they were always part of a small investigation. The condition is rare and therefore a large cohort is not possible.

Other investigations in to platelet abnormalities have given conflicting results. It is therefore difficult to form any clear understanding from the studies that have been done so far. As such, it is not possible to say that platelet adhesion occurs in the initial stages of atherosclerosis. There is circumstantial evidence to suggest that it does but no definite proof. It is likely that it does because it is known that endothelial dysfunction is involved in atherogenesis. The disruption to the endothelium results in upregulation of cell adhesion molecules and therefore leukocyte and platelet adhesion.

As such, my research proposed to investigate whether platelets adhered, either directly or indirectly via leukocytes, to the endothelium in the early stages of atherosclerosis under diabetic-like conditions. Diabetic-like conditions were chosen because diabetes is associated with accelerated rates of lesion formation and this results in increased presentation of cardiovascular disease. I hoped to be able to gain a better understanding of the processes involved in atherogenesis. By doing so, it would allow for more targeted research into drugs to reduce the onset of this condition.

To investigate this, in vitro studies were proposed, under both static and dynamic flow conditions. Endothelial cells were cultured in conditions that are associated with diabetes. These included hyperglycaemia and the presence of AGEs. Hyperglycaemia was created by incubations with high concentrations of D-glucose. The cells were treated with high glucose concentrations or with various types of AGEs as well. Selections of different purities of albumin, or haemoglobin, were glycated with D-glucose to determine whether the purities could have an effect on adhesion. Platelet adhesion (either isolated or in the presence of leukocytes) was detected by flow cytometry and immunocytochemistry. The results from this were to be compared alongside time-lapse studies to investigate the effect of dynamic flow. Cell culture studies were to be compared with those obtained when using diabetic vascular tissue (with no visible signs of atherosclerosis) to see whether the presence of other cell types could have an effect. I expected that there would be variations seen between these because, for lesions to develop, monocytes migrate through the intima. For this to occur, there are likely to be changes to the subendothelium as well as the endothelium.

If hyperglycaemia and AGEs increase platelet adhesion it is likely that they have an effect on the endothelium. Indeed, diabetes has been linked with endothelial dysfunction and reactive oxygen species (ROS) formation. High levels of glucose has been reported to result in decreased cell proliferation, viability, increased apoptosis, expression of certain cell adhesion molecules (VCAM-1, ICAM-1, E-selectin, and P-selectin), ROS and cytokine expression. These do, however, appear to be dependent on the type of glucose (although D-glucose is the only isomer found in humans and the results for this

sugar are consistent) and the origin of the cells. It would also appear that cytokine production is greater following intermittent exposure to hyperglycaemic conditions.

Although there have been a lot of investigations looking into the effect of hyperglycaemia on the endothelium there have been less looking at the effect of AGEs or a combination of high glucose and AGEs. The studies that have been reported show lots of contradictions. It would appear that the effect of AGEs is dependent on a number of factors. It is possible that these include the origin of the cells, the type of protein glycated, the concentration of AGEs, and impurities or endotoxins present in the AGE solution. Overall, however, it is possible that AGEs decrease cell proliferation, increase apoptosis, leukocyte adhesion, cell adhesion molecule expression (VCAM-1, ICAM-1, and PECAM-1), and nitric oxide expression. There does not appear to be any research showing the effect of these proteins on other ROS or on cytokine production.

Therefore, further to the platelet adhesion studies planned as part of this research, I also planned to investigate what effect diabetic-like conditions had on endothelial cells. It was hypothesized that hyperglycaemia and AGEs would lead to endothelial dysfunction (upregulation of cell adhesion molecules, cytokine production, and decreased cell proliferation and viability). These are likely to result in adhesion of leukocytes, and possibly platelets, to the endothelium, decrease lumen diameter and increase shear stress. This could lead to increased formation of foam cells by phagocytosis of ox-LDL and activate platelets, and therefore account for accelerated lesion formation seen in diabetics. Again, it was hoped that by gaining greater understanding of the processes involved it would allow for the development of drugs to reduce lesion formation and therefore the effects of morbidity and mortality following the manifestation of cardiovascular disease.

To investigate this, cell proliferation, cell adhesion molecule expression, ROS production and cytokine production experiments were to be done under diabetic conditions. This was to be achieved by culturing cells with various concentrations of glucose, and / or different types of AGEs, for a range of time periods. Normal, medium and high concentrations of glucose would then be administered. AGEs were produced both using albumin and haemoglobin. A variety of purities of albumin were glycated to investigate the effect on the endothelium. Samples were taken throughout the glycation process to allow for determination of the amount of glycation and map this to the effect on the endothelium. Cells were either cultured with medium containing high glucose or in combination with the various AGEs.

Cell proliferation was determined using cell counts and a commercial cell viability kit. Protein and gene expression of cell adhesion molecules ($\alpha_v\beta_3$, P-selectin, E-selectin as well as VWF) were to be investigated using flow cytometry, immunocytochemistry, and PCR. Both in situ and dynamic flow conditions were to be studied to see whether the high shear rates associated with vessel bifurcation had any effect on upregulation. Nitric oxide levels would then be determined by Griess assay while a more general overview of

ROS levels would be gained by looking at dihydrorhodamine oxidation. Cytokine production (IL-6, IL-8, and Fractalkine) was to be determined by using commercial ELISA kits and flow cytometry. ELISA was chosen to investigate cytokines present in the supernatant and flow cytometry for bound cytokines.

I hypothesized that a combination of hyperglycaemic conditions and AGEs would increase the levels of endothelial dysfunction. I expected to find shorter exposure to high levels would increase the expression of cell adhesion molecules, cytokines, and ROS production, while decreasing cell proliferation and viability. I thought that the greater the amount of glycation and the more impurities the greater the effect.

Overall, my research proposed to address the following research questions in the hope to provide greater understanding in this field:

- Are platelets involved in the initial stages of atherosclerosis?
- Do platelets directly adhere to the endothelium under diabetic-like conditions?
- Do platelets adhere to the endothelium via platelet-leukocyte complexes?
- Do diabetic-like conditions induce endothelial dysfunction?
- Could endothelial dysfunction in diabetics account for accelerated atherosclerosis?

There were, however, limitations to my studies. Cultured endothelial cells, although a very useful tool, have their restrictions. There are variations in characteristics between cell lines and also between batches. Immortalised cell lines tend to be poorly characterised and show non-endothelial traits, such as chromosomal instability and different responses to stimuli. Primary cells, as in this case, differ as a result of origin and donors, as mentioned previously. They are also sensitive to changes in culture conditions, be that slight variations in media constituents or conditions, and show diminished function with passage number.

I believe that in vitro studies can only show part of the picture. For a more encompassing view of the processes involved in endothelial dysfunction and atherogenesis, I think that it is important to look to human studies. Further investigations would therefore be necessary. These could include investigations similar to the PDAY trial that instead specifically investigate platelet adhesion at sites that have a high prevalence of lesion formation, adhesion at sites that have individual foam cells, and also where fatty streaks are developing.

From looking at the research available so far, I believe that it is likely that platelets are involved in lesion formation under diabetic-like conditions. The presence of both high concentrations of glucose and AGEs, over long periods of time, activate platelets and are likely to cause changes to the endothelium that result in endothelial dysfunction. I think that my research would have found that, although these diabetic-like conditions would not have significantly changed cell proliferation, that there would have been an increase

in up-regulation of cell adhesion molecules and production of cytokines and ROS. This would have resulted in monocyte adhesion, especially in the presence of activated platelets. I do, however, think that platelets are unlikely to be the initiating factor. They are not a prerequisite for foam cell formation, but are likely to accelerate atherogenesis. While the reduction in lesion formation by targeting platelet activation and adhesion would not directly result in eliminating atherosclerosis, it may well allow for a significant reduction in lesion formation in diabetics. It will also add to the general understanding of the processes involved in atherosclerosis and endothelial dysfunction. It will also assist others in future research.

7. Bibliography

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